# SOIL RESPIRATION AND NUTRIENT DYNAMICS AT A MATURE TEMPERATE WOODLAND – THE BIFoR FACE EXPERIMENT

By

ANGELIKI KOURMOULI

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School of Geography, Earth and Environmental Sciences

College of Life and Environmental Sciences

University of Birmingham

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This work is dedicated to all the victims of patriarchy and white supremacy.

To all those whose body parts, skin colour, gender performance, and expression, femininity or “lack” of masculinity, socio-economic status, and heritage were used as a justification of an act of violence.

*“The most violent element in society is ignorance.”*

*Emma Goldman*

# Abstract

The atmospheric carbon dioxide ($CO_2$) concentrations have increased approximately 50% since the beginning of the industrial era (1750), breaking the record of 415 ppm, annually. $CO_2$ is considered on of the most important contributing gases to climate change and future predictions support a continuous increase. The rate of anthropogenic emissions increases and the ecosystemic capacity to take up and store carbon (C) govern the predicted atmospheric $CO_2$ increase. The terrestrial biosphere is the principal aspect of the C cycle and climate change. Interestingly, the terrestrial biosphere is also associated with the highest uncertainties in its response to environmental change.

Forests are of global significance in the C cycle, storing approximately 860 ± 60 Pg C, with Northern temperate forests being dominant regulators, sequestering one-third of the total C uptake. The general line of thinking was that young forests could sequester C more effectively than mature forests; however recent scientific outcomes suggest that mature forests can continue sequestering C. Nonetheless, disturbances, such as increasing atmospheric $CO_2$, can alter the C storage capacity of the mature forests ecosystem. Although forest ecosystems serve as a $CO_2$ sink currently, the future projections of forest ecosystems' C storage capacity are highly uncertain.

This works aims to address the short-term belowground responses of mature temperate woodland, utilising Birmingham’s Institute of Forest Research Free Air $CO_2$ Enrichment (BIFoR FACE) facility. BIFoR FACE is the only large-scale climatic experiment in the Northern Hemisphere, studying a mature forest ecosystem's responses under the atmospheric $CO_2$ scenario of 2050. Subsequently, this works aims to provide an insight into the belowground interactions of three biogeochemical

cycles (C, N, and P) and the potential shifts under elevated $CO_2$ using high-resolution frequency measurements for the first time in the history of forest FACE experiments.

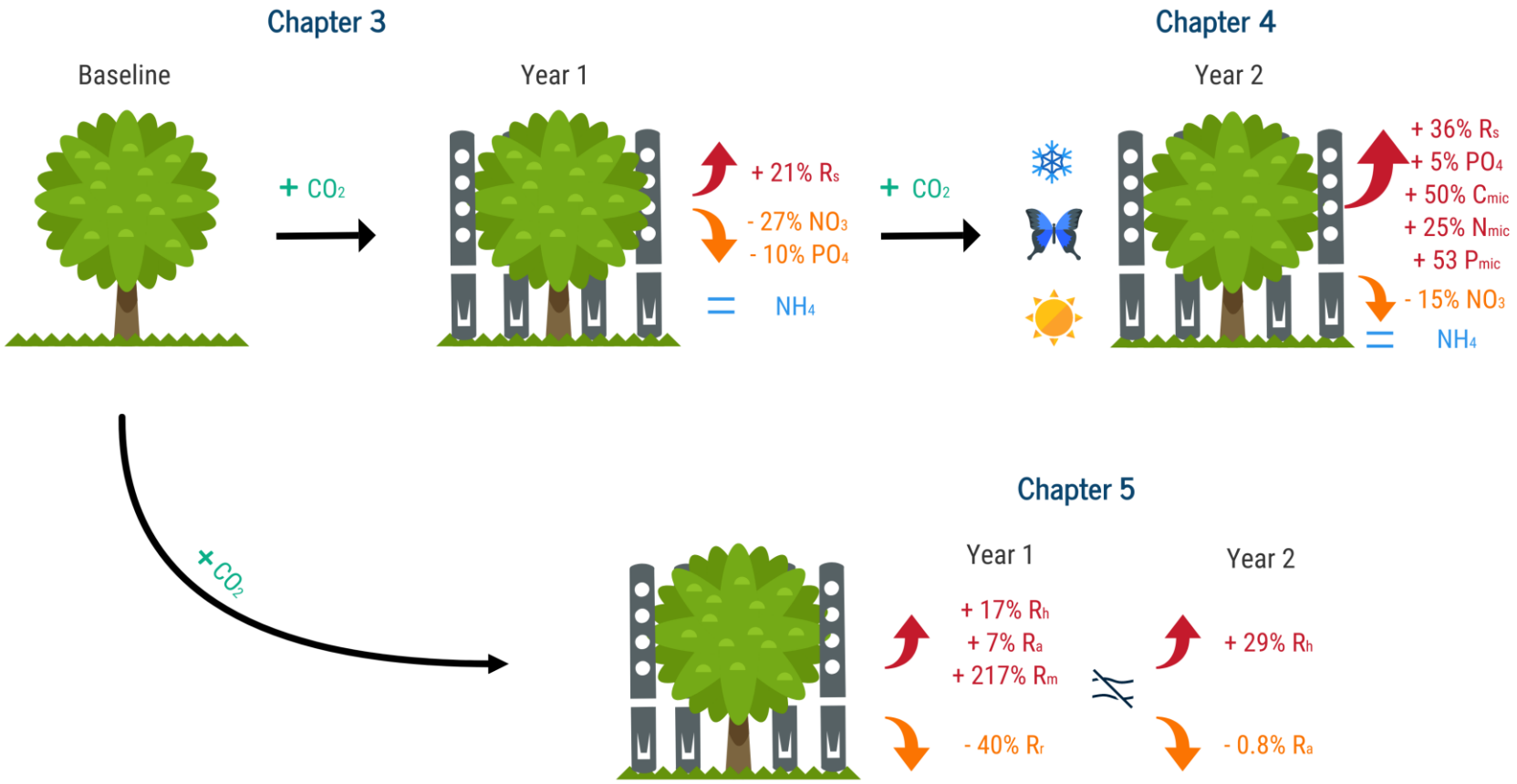
During the first year of $eCO_2$ enrichment, a 21% increase was observed in soil respiration, on an annual average, releasing approximately 283 g C $m^{-2}$ $y^{-1}$ more than in ambient $CO_2$. Moreover, $NO_3^-$-N availability showed a prompt and sustained decrease, up to 37% in monthly averages, in $eCO_2$ arrays, whereas $eCO_2$ did not affect $NH_4^+$-N and $PO_4^{3-}$-P availability in the soil.

During the second year of $eCO_2$ enrichment, we also had the opportunity to monitor a series of extreme events (Beast from the East, winter moth outbreak, and 6-week summer heatwave), alongside the belowground responses to $eCO_2$. The drought caused by the heatwave had a severe effect on soil respiration, suppressing soil respiration by 47 and 40% in ambient and $eCO_2$ arrays, respectively, compared with the previous year. $eCO_2$ dampened the extreme event's impact by increasing soil respiration by ~36% in $eCO_2$ arrays, releasing approximately 259 g C $m^{-2}$ $y^{-1}$ more to the atmosphere. A winter moth outbreak led to a short-lived manifold increase in soil nutrient availability during autumn for all three nutrients, with the effect being more prominent under $eCO_2$.

Moreover, we attempted to partition soil respiration to its respiratory sources (autotrophic, heterotrophic, root, and hyphal respiration), *in situ*, during the first two years of $eCO_2$ enrichment. $eCO_2$ stimulated all respiratory components, except for root respiration, during the experiment's first year. This contribution was persistent during the second year through the series of extreme events. The heterotrophic component was the main contributor to soil respiration in both years. However, the respiratory

components' sensitivities to abiotic factors (soil temperature and moisture) were altered under the combined effects of $eCO_2$ and extreme events.

# Content & Structure of the Ph.D. thesis



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# List of Abbreviations

BIFoR – Birmingham's Institute of Forest Research

°C – degrees of Celsius

C – Carbon

cm - centimetre

$CO_2$ – Carbon dioxide

$Ca_3(PO4)_2$ – Calcium phosphate

$eCO_2$ – Elevated $CO_2$

EE – Extreme events

EM – Ectomycorrhizal fungi

FACE – Free Air $CO_2$ Enrichment

$F_c$ - soil $CO_2$ efflux rate

$FePO_4$ – Iron phosphate

GPP – Gross primary productivity

H – Hydrogen

IEM – Ion-exchange membrane

km – Kilo metres

$Mg_3(PO_4)_2$ – Magnesium phosphate

Mha – Mega hectares

mm - Millimetre

mol - Mole

N – Nitrogen

$N_2$ – Dinitrogen

$N_2O$ – Nitrous oxide

$NH_4^+$ – Ammonium

$NO_2^-$ - Nitrite

$NO_3^-$ – Nitrate

NO – Nitrogen oxide

NEP – Net ecosystem productivity

NPP – Net primary productivity

O – Oxygen

P – Phosphorus

$P_0$ - Pressure

Pg – Pentagrams

$PO_4^{3-}$ - Phosphate

ppm – parts per million

$Q_{10}$ – Temperature sensitivity

R - Gas constant

$R_a$ – Autotrophic respiration

$R_e$ – Ecosystem respiration

$R_h$ – Heterotrophic respiration

$R_m$ – Hyphal respiration

$R_r$ – Root respiration

$R_s$ – Soil respiration

S - Soil surface area

$T_0$ - Air temperature

$T_s$ – Soil temperature

V - volume

VWC – Soil moisture

$W_0$ - Water vapour mole fraction

$(\partial C')/\partial t$ - Rate of change in water-corrected $CO_2$ mole fraction

µ - micro

# Chapter 1: Introduction

This Chapter introduces fundamental knowledge of the importance of forested ecosystems and the forests' carbon cycle. An introduction to soil respiration and its controlling factors are included and the nitrogen and phosphorus biochemical cycles. Following this, an introduction to climate change and, more specifically, on elevated atmospheric carbon dioxide and a brief literature review on elevated carbon dioxide impacts on soil respiration and nutrient dynamics. Finally, previous FACE experiments, the BIFoR FACE experiment, and the thesis' layout are discussed.

## 1.1 Importance of Forest Ecosystems

The term forest ecosystem includes the living organisms of the forest (plants, animals, microbes, fungi) and their interaction with their environment's chemical and physical components. It extends vertically upward to the atmospheric layer encasing forest canopies (can be up to 120 m height; Tao *et al.*, 2016) and downward to the lowest soil layers affected by roots and biotic processes (up to 68 m; Canadell *et al.*, 1996).

Forest ecosystems are of high importance to natural systems and society through the services they provide. The services are classified in the following categories: *provisioning services*, such as food, medicinal, and forest products; *regulating services* that affect floods and climate, through energy, water, carbon dioxide, and other chemical species with the atmosphere; *supporting services*, such as nutrient cycling, soil formation and refuges for biodiversity; and *cultural services* that include aesthetic, spiritual, educational and recreational uses (Hassan, Scholes and Ash, 2005). Another vital ecosystem service that is often overlooked is that for millennia, forests have

provided a home for millions of people worldwide, and their survival is highly dependent on the forest (Soussan, Shrestha and Uprety, 1995).

Forests currently cover ~42 million $km^2$, accounting for almost one-third of the land surface, storing ~45% of terrestrial carbon (C), and contributing ~50% of terrestrial net primary production (Field and Raupach, 2004). In the 1990s, the terrestrial C sink was estimated around 2.6 Pg C $year^{-1}$ (Nabuurs *et al.*, 2007), while more recent analyses estimate the net terrestrial C sink in the range of 2 to 3.4 Pg C $year^{-1}$ (Khatiwala, Primeau and Hall, 2009; Le Quéré *et al.*, 2009). Forests can veil the high albedo of snow due to their low surface albedo (Jin *et al.*, 2002), which exacerbates the planet's warming through increased land solar heating. They can also cool the climate since they regulate the hydrologic cycle through evapotranspiration (Baldocchi and Xu, 2007). Thus, forests are of high significance in the global C cycle by absorbing carbon dioxide ($CO_2$) and storing it within the ecosystem.

Among different terrestrial ecosystems, forest soil contains more than two-thirds of the global soil organic C, being the highest C-rich ecosystem among the different land used-based ecosystems (Ahmed, 2018). The current global C stock in the world's forests is estimated to be 861 ± 66 Pg C, with 383 ± 30 Pg C in the soil, 363 ± 28 Pg C in live biomass (above and below ground), 73 ± 6 Pg C in deadwood and 43 ± 3 Pg C in the litter. Geographically, 55% of the global C stock is stored in tropical forests, 32% in boreal, and 14% in temperate forests (Pan *et al.*, 2011).

Humans can affect forest ecosystems through their activities by influencing the composition, cover, age, and density of the vegetation, by altering the kinds of stands present and their spatial arrangement, influencing the movement of wind, water, animals, and soils, by introducing by-products into the atmosphere that may fertilize

or harm the forests. Moreover, the continuous consumption of fossil fuels has led to an increase in the atmospheric $CO_2$ concentration, altering the biogeochemical cycles inducing climate change. The higher demand for forest products is exhilarating the transfer of products from one country to another, introducing non-native species and associated pests, threatening native forests and fauna.

### 1.1.1 Temperate Forests

Temperate forests are located in the mid-latitudes and form a patchwork of discontinuous forests on five continents surrounded by prairies, steppes, or deserts. They are characterized by species diversity, warm-temperate mixed broadleaved species, cold-temperate coniferous, evergreens, deciduous broadleaved, and mixed broadleaved/coniferous species. All temperate forests have been directly impacted by human beings, except those in major mountain areas, since extensive forest areas were cleared for agriculture since the 12$^{th}$ century (Wisniewski and Sampson, 1993). It is uncommon for temperate forests to cover more than 40% of the land area since they tend to be surrounded by agricultural land and urban areas. The temperate forest biome area has been increasing since the beginning of the 20$^{th}$ century, both due to the ecosystem's natural recovery after agriculture abandonment and increased newly planted forests (Fang *et al.*, 2001; Birdsey and Lucier, 2006; Kauppi *et al.*, 2006).

Temperate forests cover 767 Mha of the global land surface and are currently storing 119 ± 6 Pg C (Pan *et al.*, 2011). They have 42% of C stored in biomass and 48% in soil. For the past two decades, they have been an increasing carbon sink, absorbing ~0.72 ± 0.1 Pg C year$^{-1}$, primarily due to an increase in forest area and biomass density (Birdsey, Pregitzer and Lucier, 2006; Kauppi *et al.*, 2006). Notably, China's and U.S.

C sink showed 33 and 34% increase, respectively, due to increased forest area, growth of immature forests, and increased area of newly planted forests (Fang *et al.*, 2001; Birdsey, Pregitzer and Lucier, 2006), $CO_2$ fertilization and N deposition (Pan *et al.*, 2009). However, the European C sink was stable for the past decades (Ciais *et al.*, 2008).

### 1.1.2 Tropical Forests

Tropical forests are located around the equator, in three continents, and are the most complex biome in terms of vegetation structure and species richness. Climatically, these regions are characterized by high moisture and temperature all year round, creating an ideal environment for broadleaved evergreen types. Due to the high precipitation rates and the fact that these forests are grown on bedrock, most nutrients are leached away. These forests have undergone many land-use changes over the centuries, with many mature forests converted to logged forests, permanent agriculture, and pasture or shifting cultivation. The rates of land-use change depend on social, economic, and political factors.

Tropical forests cover 1949 Mha, accounting for the largest global forest biomes and currently storing 471 ± 93 Pg C. Tropical forests have 56% of C stored in biomass and 32% in soil. For the past two decades, tropical forests have been a decreasing C sink, absorbing ~2.8 ± 0.7 Pg C $year^{-1}$ (Pan *et al.*, 2011), equivalent to more than half of the total C sink in established forests. The sink reduction observed was due to deforestation of the intact forest area (Houghton, 2007) and severe Amazon drought (Phillips *et al.*, 2009).

### 1.1.3 Boreal Forests

Boreal forests form a circumpolar belt through northern Eurasia and North America. Its northern boundary is formed with subarctic woodlands containing treeless patches and stunted forest stands that form the boundary between the boreal forests and the tundra zones. The southern boundary is dominated by temperate deciduous forests where oceanic influences moderate climate and arid steppe or prairie. There is no defined boundary between the boreal and the temperate forests; instead, there is a transitional zone with mixed-species stands or a patchwork of temperate deciduous forests and boreal conifers. These ecosystems are characterized by short growing seasons and low mean temperatures resulting in a forest cover dominated by coniferous vegetation.

Boreal forests cover 1135 Mha of the Northern Hemisphere and currently store 272 ± 23 Pg C. Boreal forests have 20% of C stored in biomass and 60% in soil. The past two decades have been a consistent carbon sink, absorbing ~0.5 ± 0.1 Pg C $year^{-1}$ (Pan *et al.*, 2011). Mainly, Asian Russia, which is the most significant boreal sink, exhibited no overall change, regardless of the increased emissions from wildfire disturbances. Moreover, in European Russia and Northern Europe, there was a 35% sink increase due to increases in forest areas after agricultural abandonment, reduced harvesting, and forest age structure changes to more productive stages (Kauppi *et al.*, 2010; Schepaschenko *et al.*, 2015). Conversely, in Canada, the C sink was reduced by 50% due to biomass loss from intensified wildfires and insect outbreaks (Stinson *et al.*, 2011).

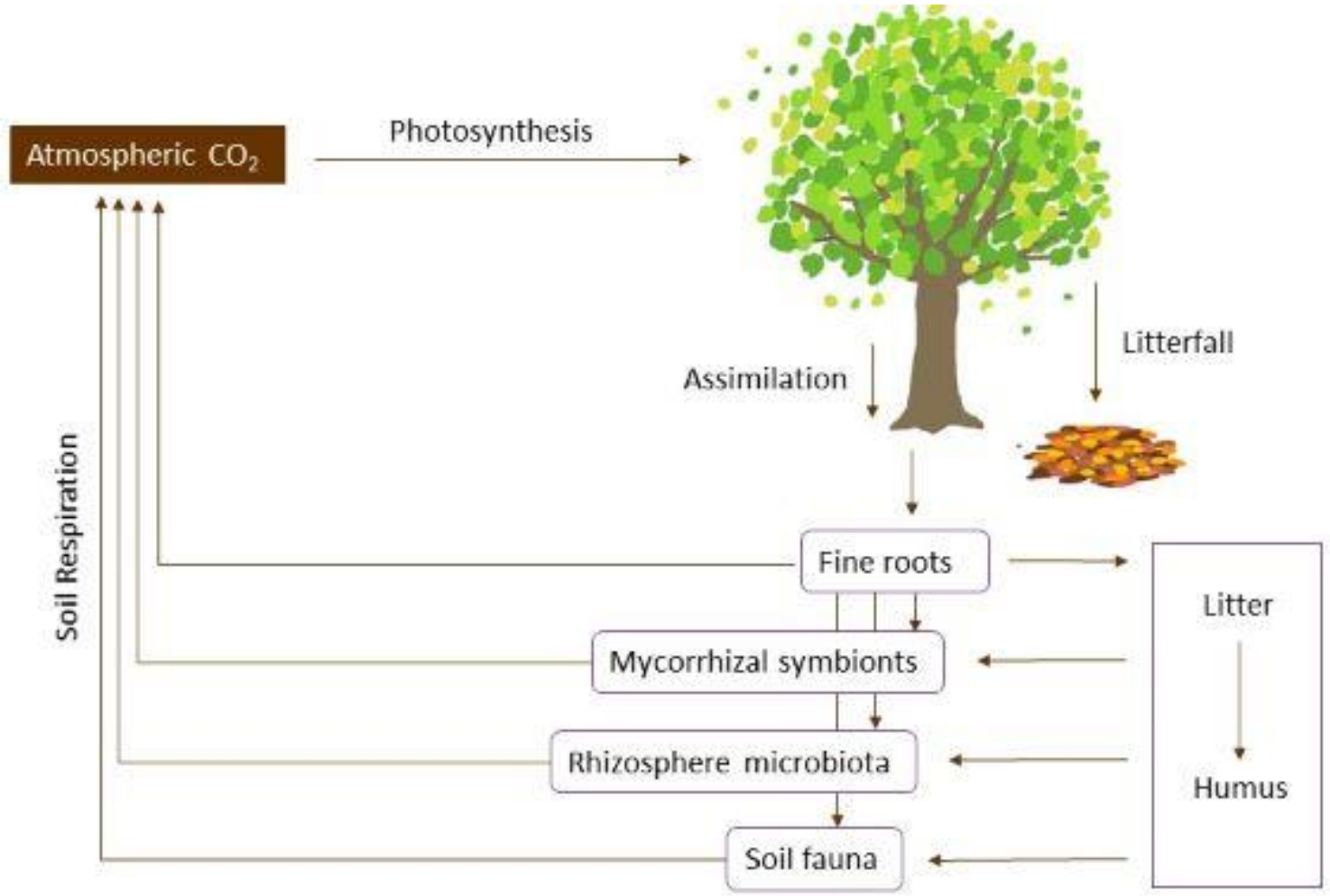


***Figure 1- 1:*** *Simplified schematic diagram of the terrestrial C cycle in a forest ecosystem*

## 1.2 Forest Carbon Cycle

In the forest ecosystem, carbon enters the system (Fig. 1-1) when plants assimilate atmospheric $CO_2$ through photosynthesis into reduced sugars (Reaction 1). $CO_2$ enters the plants by diffusion through leaves' stomata and then is transferred to photosynthesis sites. Photosynthesis is the process by which plants convert $CO_2$ to carbon products and takes place within cells containing chloroplasts. This process is restricted by physical and biochemical processes and involves reactions that require both light and dark.

$$CO_2 + H_2O \text{ (+ light energy)} \rightarrow CH_2O + O_2 \qquad \text{Reaction 1}$$

$$CH_2O + O_2 \rightarrow CO_2 + H_2O \text{ (+ energy)} \qquad \text{Reaction 2}$$

Approximately half of the gross photosynthetic products (GPP) are used up by plants through autotrophic respiration ($R_a$) for synthesis and maintenance of living cells, releasing $CO_2$ back to the atmosphere (Reaction 2). The remaining photosynthetic products go into foliage, branches, stems, roots, and plant reproductive organs, potentially stored on short, medium, and long term in the forest. This rate is called net primary production (NPP) and is the difference between GPP and $R_a$ (Equation 1).

$$NPP = GPP - Ra \qquad (1\text{-}1)$$

Therefore, NPP is the amount of C and energy that enters the ecosystem. NPP provides energy for all biotic processes, such as decomposer organisms' activity that recycle nutrients essential for supporting primary production and is the product of multiple interactions among elements, organisms, and the environment. Thus, small changes in these processes can significantly impact NPP and shift the forest's ability from a C sink to a C source.

Eventually, plants will shed leaves and roots; the dead organic matter forms a substrate that supports microbes, fungi, and animals, which, through their metabolic processes, release $CO_2$ back into the atmosphere ($R_h$). Some detritus decompose quickly (respired to the atmosphere, Reaction 2), and some are modified to carbon that decomposes more slowly. The rate at which the decomposition will take place depends on the dead tissues' chemical composition and environmental conditions. The turnover of detritus and microbial biomass is less than ten years, whereas modified soil organic C turnover can be up to centuries. A small amount of the modified carbon is further converted to much more resistant to decomposition compounds, creating a recalcitrant carbon pool.

The difference between NPP and $R_h$ determines how much carbon is lost or gained by the ecosystem in the absence of disturbances. Annually, undisturbed forest ecosystems exhibit a small net gain of C, and this represents the net ecosystem production (NEP) and is the difference between the GPP and the total ecosystem respiration ($R_e$) (Equation 2).

$$NEP = GPP - Re \tag{1-2}$$

In forests, the major contributors of the soil C stock are the decomposed plant tissues, above and belowground, and the root exudates; however, the fine root and mycorrhizal turnover on soil C storage are considered to be more crucial than the C in the aboveground litter (Rasse, Rumpel and Dignac, 2005). Microbial biomass, bacteria, archaea, and mycorrhizal fungi contribute to soil organic carbon stock by releasing stored C through decomposition and respiration, and biomass production.

## 1.3 Soil Respiration

Soil contains almost double the amount of C of the vegetation and atmosphere combined (van Groenigen *et al.*, 2015), and soil respiration ($R_s$) is the second-largest flux of $CO_2$ in the terrestrial ecosystems, linking soil and atmospheric C, through the C cycle (Ahmed, 2018). The soil C pool is approximately four times higher than the atmospheric pool; thus, minor changes in the flux of $CO_2$ from soil to the atmosphere may significantly impact the balance of the atmospheric $CO_2$ (Luo and Zhou, 2006).

$R_s$, the $CO_2$ efflux from the forest soil, is a combination of autotrophic root activity and associated rhizosphere organisms, heterotrophic bacteria and fungi, and soil fauna activity (Fig. 1-2). $R_s$ is divided into autotrophic respiration ($R_a$), which is the $CO_2$ produced from the metabolic activity for root growth and maintenance; and heterotrophic respiration ($R_h$), which is the $CO_2$ produced from the decomposition of organic matter used as energy source for microbial communities (Savage *et al.*, 2013).

Usually, the ectomycorrhizal (EM) fungi respiration ($R_m$) is included in the $R_a$, due to the difficulty in separating the root from the EM, *in situ*, leading to overestimation of the $R_a$ and underestimations of the $R_h$ (Tomè *et al.*, 2016). It is essential to highlight that each soil component (roots, fungi, free-living microbes, and soil animals) has a unique dependence on substrate quality and abiotic factors and may exhibit different behaviours under a change in their environment or climate change (Wei, Weile and Shaopeng, 2010; Ma *et al.*, 2014). Thus, it is crucial to quantify the flux from each component to understand better the drivers and the processes involved and make more accurate model predictions.

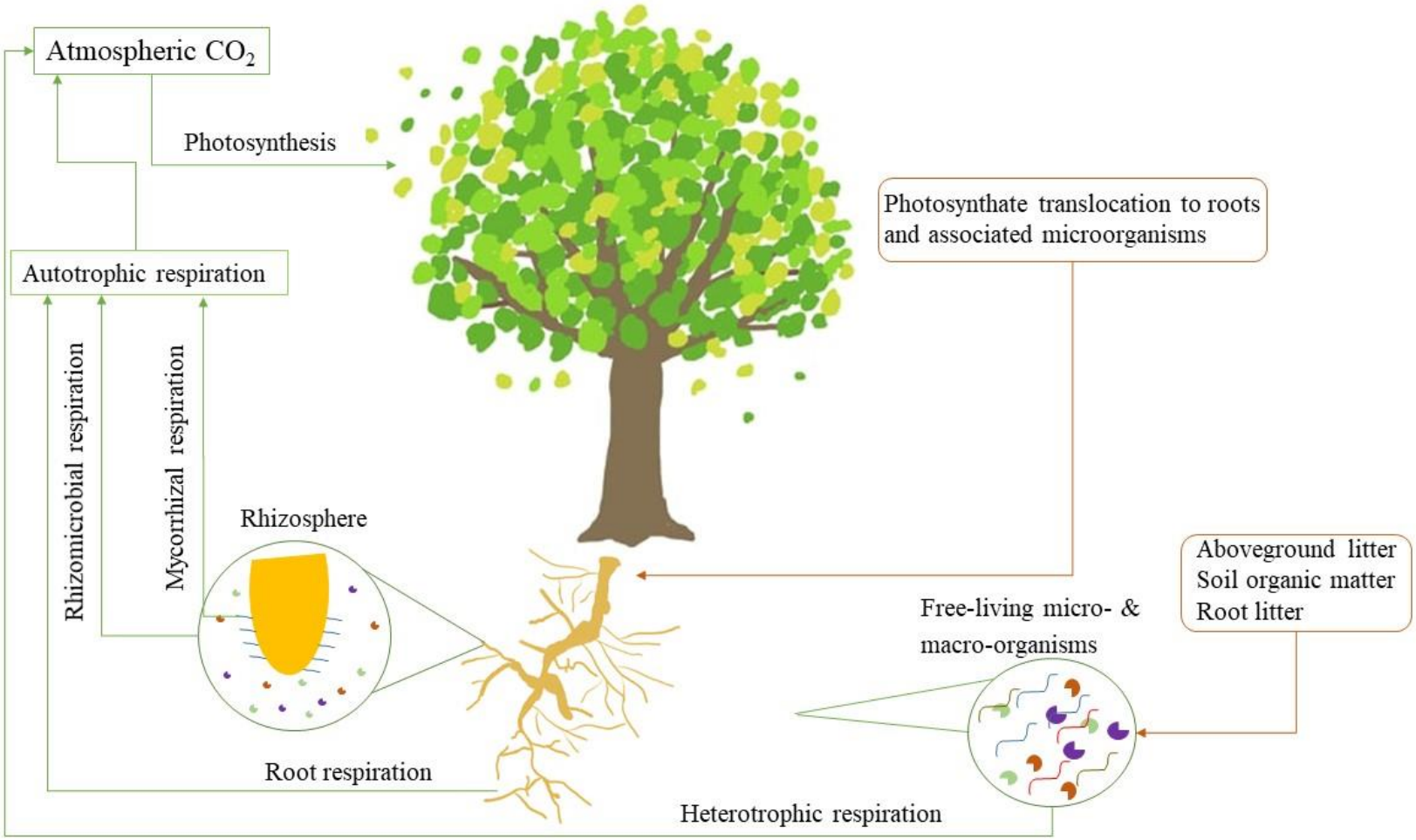


***Figure 1- 2:*** *Simplified schematic diagram of soil respiration sources in a forest ecosystem*

Mycorrhizal fungi form a symbiotic relationship with the plant roots, depending on the roots for their C needs, and in exchange, the fungi provide essential nutrients to the plants. By having a smaller diameter than tree roots, fungal hyphae can access films of water on soil particles that roots cannot. Specifically, in the temperate regions, roots are in symbiosis with EM fungi, which form a sheath around the fine roots' tips, increasing the root system efficiency. Due to this mutual relationship, EM do not have to compete with other soil heterotrophs for energy sources; instead, they acquire essential sugars directly from the roots (Brady and Weil, 2017).

At the beginning of the growing season, little to no decomposable material is added to the soil via litterfall; thus, $R_h$ rates are low. As the growing season progresses and litterfall increases, more decomposable material is added to the soil, and $R_h$ rates increase. $R_a$ and $R_h$ have similar seasonal behaviour, with $R_a$ increasing slightly during the growing season than $R_h$ (Högberg *et al.*, 2001). Each soil component's relative contributions vary hugely, from 10% to 90%, depending on the type of ecosystem, the time of the year, and the measurement technique (Hanson *et al.*, 2000).

## 1.3.1 Controlling factors on soil respiration

Various factors control $R_s$ in a complex way. The most influential factors in temperate ecosystems (Ryan and Law, 2005) are soil moisture, soil temperature, and substrate supply (Suseela *et al.*, 2012).

#### 1.3.1.1 Soil Moisture

Soil moisture is a significant control on $R_s$ because it can alter the soil oxygen levels, which both $R_a$ and $R_h$ are sensitive to. High water content inhibits both aerobic root

respiration and microbial decomposition (Brady and Weil, 2017) by filling up the soil pores and preventing oxygen (O) diffusion into the soil. It alters the $CO_2$ movement in the soil and acts as a barrier for diffusion (Pumpanen *et al.*, 2008). Moreover, high water content can trigger an increase in $R_s$ in the short term, e.g., after rainfall events pulse responses (Gaumont-Guay *et al.*, 2006; Yan *et al.*, 2014). Low water content limits the substrate diffusion for $R_h$ and causes Ra's desiccation stress (Davidson and Janssens, 2006; Van der Molen *et al.*, 2011). Nevertheless, soil fungi are better adapted to water stress than bacteria or soils' microfauna (Swift, Heal and Anderson, 1979).

It is difficult to interpret the effect of soil moisture on $R_s$ due to other factors that vary seasonally, such as the seasonal covariance of photosynthetic rate, precipitation, litterfall, root, and microbial activity (Jassal *et al.*, 2008). However, studies have suggested that the optimal conditions for $R_s$ are at intermediate water content levels (Davidson, Belk, and Boone, 1998) and appear to be a primary driver of $R_s$ when the volumetric soil moisture content is less than 20% (Jassal *et al.*, 2008; Almagro *et al.*, 2009).

#### 1.3.1.2 Soil Temperature

While temperature is a critical driver for $R_s$, and especially for $R_h$ (Suseela *et al.*, 2012), as with soil moisture, there is a difficulty when interpreting the effect of temperature on $R_s$ due to other important factors such as accessibility and supply of organic substrate to microbes that limits $R_h$ (Davidson and Janssens, 2006), and long term substrate limitations that inhibit $R_a$ (Schindlbacher *et al.*, 2009).

However, the $R_s$ sensitivity to temperature is often described with the temperature coefficient $Q_{10}$, a factor by which respiration rates change by a temperature rise of 10 °C. Typically, it is assumed that the respiration rates double for every 10 °C in the temperate regions (Davidson and Janssens, 2006; Noh *et al.*, 2015), but seldom studies show differences in short and long term responses of $R_s$ to changes in temperature (Larigauderie and Körner, 1995; Atkin *et al*., 2000). Since $Q_{10}$ is calculated via seasonal temperature variations, it is challenging to distinguish the temperature effect from other seasonal factors, such as carbon allocation to roots, solar radiation, and moisture (Jia *et al.*, 2013; Wang *et al.*, 2014; Han *et al.*, 2014). In the literature, $R_a$ is described twice as sensitive ($Q_{10}$ ~4) as $R_h$ ($Q_{10}$ ~2) (Boone *et al.*, 1998; Epron *et al.*, 2001), but has been criticized since other seasonal factors have been overlooked (Högberg *et al.*, 2001; Bhupinderpal-Singh *et al.*, 2003; Ruehr and Buchmann, 2009; Schindlbacher *et al.*, 2009). There are studies that exhibit a lack of response of $R_a$ and/or $R_h$ to temperature (Högberg *et al.*, 2005; Heinemeyer *et al.*, 2007).

#### 1.3.1.3 Soil Organic Matter Content

$R_s$ vary significantly in different biomes because vegetation dictates the soil microclimate and structure and the quality and quantity of the substrate supply (Raich and Tufekcioglu, 2000; Mitra *et al.*, 2014). Assuming that no other factors are limiting, adding soil organic matter increases the $R_h$ since it supplies soil heterotrophs with essential elements for their metabolism. Thus, the overall $R_s$ increases. According to Raich and Tufekcioglu (2000), $R_s$ increases almost proportionally with readily degradable soil organic matter. Apart from colder climates, decomposition is inhibited

due to colder temperatures, resulting in accumulating large amounts of soil organic matter (Schlesinger and Andrews, 2000).

## 1.4 Soil Nutrients

Various mineral/elemental cycles in a forest are strongly linked with the C cycle. Changes in the C cycle might shift other mineral cycles in the soil and negatively impact plant growth or climate change. Chemical elements and minerals that are essential or beneficial for plants' life-cycle are defined as nutrients. Plants respire the carbon acquired from photosynthesis to release energy and recycle nutrients internally from older to newer tissues. Heterotrophic and organisms associated with plants via symbiotic relationship rely on roots for their C supply to cover their energy demands and provide essential nutrients for the plant in biochemical forms.

Plants require C, hydrogen (H), and O (non-mineral elements) obtained via atmosphere and water. As described in section *1.2 Forest Carbon Cycle*, C is fixed via photosynthesis and is the primary component of many biological molecules. H is obtained almost exclusively via water and plays a crucial role in photosynthesis and respiration. O is obtained via the atmosphere or water and is also pivotal for respiration.

The rest of the essential elements required for plant maintenance and growth are obtained from the soil and are separated into two categories. Nutrients such as nitrogen (N), phosphorus (P), sulfur (S), potassium (K), calcium (Ca), and magnesium (Mg) are called macronutrients because the plants acquire them in large amounts and are essential for primary plant biological functions. Nutrients such as iron (Fe), copper (Cu), zinc (Zn), molybdenum (Mo), manganese (Mn), boron (Bo), chlorine (Cl), and

nickel (Ni) are called micronutrients because the plants require smaller amounts. Each of these elements, essential not only for plant growth but also for microbial metabolic processes, has unique chemistry in the soil, its particular relationship to lithology and atmospheric inputs, and its rate of circulation through vegetation. Alterations in inputs/outputs of biochemical cycles can lead to nutrient limitations and affect the soil's sequestering carbon capacity.

Given the importance of C, N, and P in an ecosystem, the cycles of these elements are tightly coupled and interact dynamically with each other (Fig. 1-3), thus understanding the global C balance requires scrutinised monitoring of all three cycles together in order to make understand the potential shifts in those cycles and extrapolate for the future terrestrial C feedbacks to the atmosphere. C uptake and sequestration can be profoundly constrained by N and P limitations; thus, the current hypothesis of enhanced forest growth and C storage might be highly overoptimistic, depreciating the rate and extent of climate change (Houghton, 2007).

Nutrient availability is dependent on soil age. Very young soils (formed from recent volcanic eruptions) have very high total P available (~800 mg $kg^{-1}$), while total N is very low (<5 mg $kg^{-1}$). Total P decreases with soil age reaching 30 mg $kg^{-1}$ in ancient weathered soils. Total N reaches a peak (8000 mg $kg^{-1}$) to decrease again as the soils are becoming more weathered (Lambers *et al.*, 2008). Precipitation and temperature are the critical factors for soil development since they influence root metabolic processes, microbial activity, and erosion.

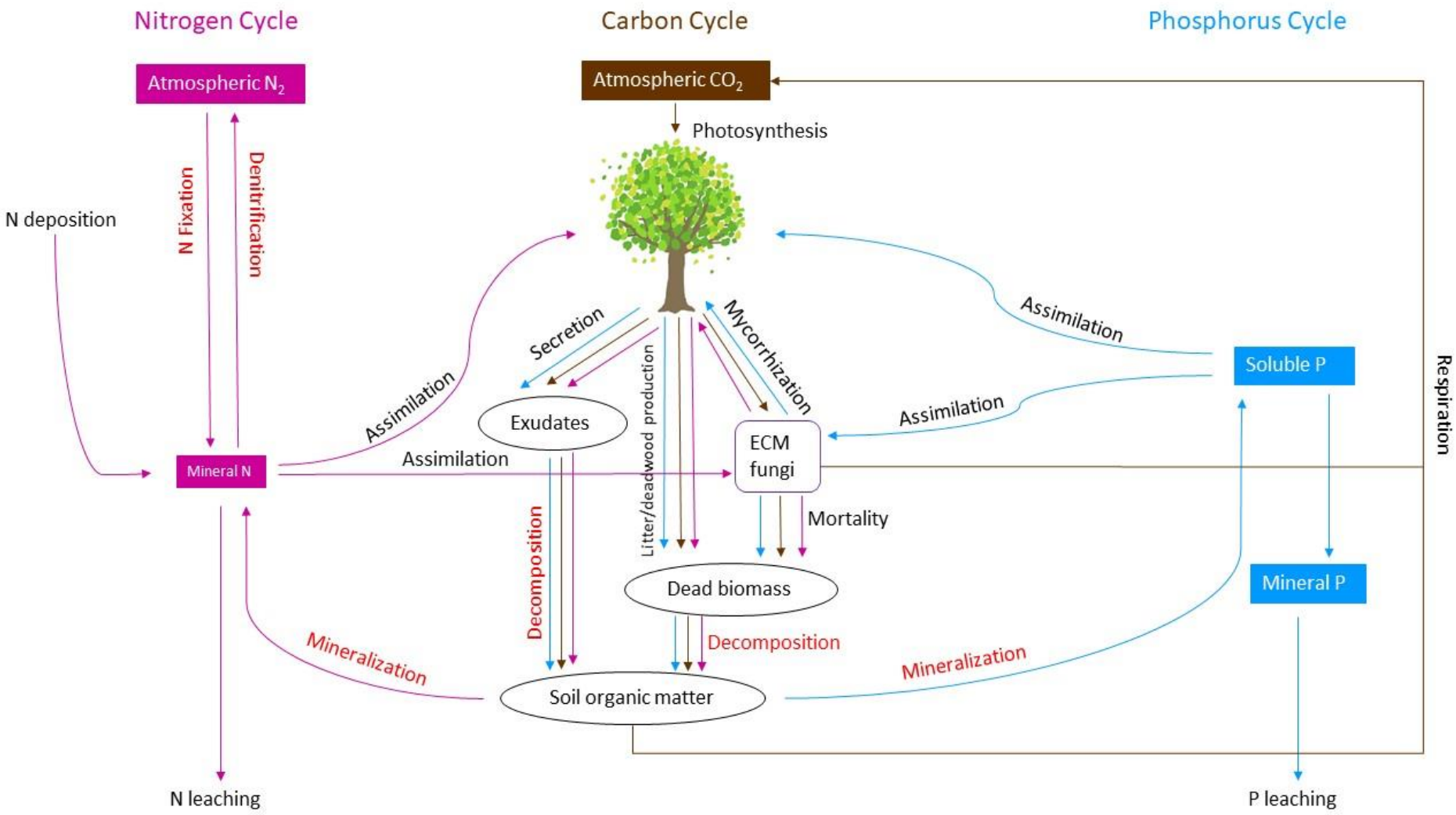


***Figure 1- 3:*** *Simplified schematic diagram of the three most important terrestrial biogeochemical cycles (C, N, and P) in a forest ecosystem*

All three elements (C, N, and P) are transformed between organic and inorganic forms (Fig. 1-3). Plants uptake the inorganic forms of nutrients and convert them to organic forms, which are assimilated by the free-living soil heterotrophic microorganisms. Once the free-living heterotrophs die, saprotrophs will decompose their remains and release inorganic nutrient forms back to the soil, making them available to plants again—this way, a continuous nutrient supply is ensured across the ecosystem.

Variations in the terrestrial ecosystems' efficiency to uptake nutrients might be due to species variations among sites or even within a species due to different nutrient availability responses. In forests, an essential process that drives the nutrient availability and requirements is the annual nutrient circulation within the ecosystem through the decomposition of dead material. Hence, changes in the biogeochemical cycles can lead to significant imbalances in the system. Studies have shown that temperate deciduous forests have higher decomposition rates than coniferous forests, mainly due to lower leaf turnover rates in coniferous forests (Cole and Rapp, 1981). However, coniferous forests have lower leaching losses than deciduous (Vitousek, 1982), resulting in greater nutrient-use efficiency in coniferous forests than deciduous forests. In temperate ecosystems, nutrient availability is one of the most critical drivers for tree growth and ecosystem function (Elser *et al.*, 2007).

Three mechanisms achieve nutrient uptake by the plant roots: the diffusion of ions toward the root surface when uptake rates exceed supply; the mass flow of ions with the movement of soil water due to transpiration; the growth of roots and mycorrhizal hyphae into the soil. Often, the plant demand for N and P exceeds delivery by mass flow; thus, diffusion is the dominant mechanism that provides these macronutrients to the plants (Eissenstat and Rees, 1994). Several factors control the plants' nutrient uptake, such as the rate of photosynthesis, soil respiration, root uptake capacity,

nutrient reabsorptions during leaf senescence, differences in leaching, and inherent differences in the rate at which photosynthate is incorporated in plant growth (Schlesinger, 1991). Available nutrient concentrations at the absorbing root surface, total root surface area, and root distribution in the soil are factors that can limit nutrient uptake (Lambers *et al.*, 2008).

However, plants have developed multiple strategies to acquire more nutrients, especially in heavily weathered soils that are nutrient-impoverished. These strategies involve developing long root hair, accelerated root growth, localised accelerated root growth in nutrient-enriched zones, increased exudation of carboxylates, forming symbiotic relationships with mycorrhizal hyphae or root nodules, and specialised root structures, such as cluster roots (Lambers, Chapin and Pons, 2008). Plants do not depend only on one strategy in order to acquire the essential nutrients. Instead, they adopt a combination of strategies to optimise their nutrient uptake via scavenging and mining (Lambers *et al.*, 2003).

### 1.4.1 Nitrogen Cycle

Nitrogen (N) has a very complex biogeochemical cycle on the Earth. It has an essential role in the enzymes which control the biochemical reactions in which C is reduced and oxidized. A significant number of biochemical transformations of N are possible since its valence state has a range of -3 (reduced N in ammonia) to +5 (oxidised N in nitrate ions). Numerous microorganisms take advantage of that potential for transformations between these oxidation states and use the energy released by the changes in redox potential to sustain their metabolic processes (Rosswall, 1982). The most abundant and least reactive form that N can be found at the Earth's surface is the gaseous one

($N_2$). Various processes convert $N_2$ to forms that biota can use and ultimately return it to the atmosphere as $N_2$.

*Biological N fixation* is the process where $N_2$, a highly unreactive gas, is converted to $NH_3$ via a minority of specialised microorganisms which can metabolise $N_2$ to $NH_3$ for their nutritional purposes. This process is crucial for the ecosystems since plants and organisms' plurality depends on it for $NH_3$, although it is indirectly available to them (Freedman, 2018). There are other pathways in which N is fixed and becomes available to the ecosystem, such as $NH_4^+$ and $NO_3^-$ via wet and dry atmospheric deposition and direct uptake of NO and $NO_2$ by plants; however, when compared to biological N fixation are considered secondary.

Most of the N in forest soils is fixed in organic compounds that cannot be directly used by plants (Fig. 1-4). Soil heterotrophic microbes decompose dead organic material on the forest floor (*decomposition*), mineralizing organic N (*mineralization*). During these processes, soil microbes respire $CO_2$, while nutrients are retained in microbial biomass. Through mineralization, ammonium ions ($NH_4^+$) are released, and this process is called *ammonification*. Some of the $NH_4^+$ produced is taken up by plant roots or immobilized by microbes (*immobilization*). As N is often a limiting nutrient for microbial growth, N immobilization is rapid and can be significant (O'Connell, 1988), dominating N processing on the soil surface, while mineralization dominates in the deeper forest floor. N is released as $NH_4^+$ from dead microbial tissue (Van Veen *et al.*, 1987).

Furthermore, $NH_4^+$ can be lost to sorption (fixing on clay minerals) or undergo *nitrification*. Nitrification is the process in which $NH_4^+$ is oxidized to $NO_3^-$ and is a two-steps process. Firstly, $NH_4^+$ is converted to $NO_2^-$ via *Nitrosomonas* bacteria, which is

oxidised to $NO_3^-$ by *Nitrobacter* bacteria. The second step is much more rapid than the first step, and both bacterial types are sensitive to acidity. Thus plants growing in acidic soils must be able to utilise $NH_4^+$ as an N source. Some heterotrophic organisms can also convert organic N directly to $NO_3^-$ (Waring and Running, 2007). Subsequently, $NO_3^-$ will be uptaken by plant roots or immobilized by microbes, although soil organisms often show a distinct preference for $NH_4^+$ (Jackson, Schimel and Firestone, 1989). Moreover, $NO_3^-$ can be lost from the system through leaching or undergo *denitrification*.

Denitrification is the process in which $NO_3^-$ is converted to $N_2O$ and $N_2$, facilitated by soil aerobic heterotrophs. Contrary to nitrification, which is facilitated by specific microbial species, denitrification is facilitated by a wider bacterial variety and requires anaerobic conditions. Denitrification is accelerated under high $NO_3^-$ amounts in soil and is considered an N fixation offset.

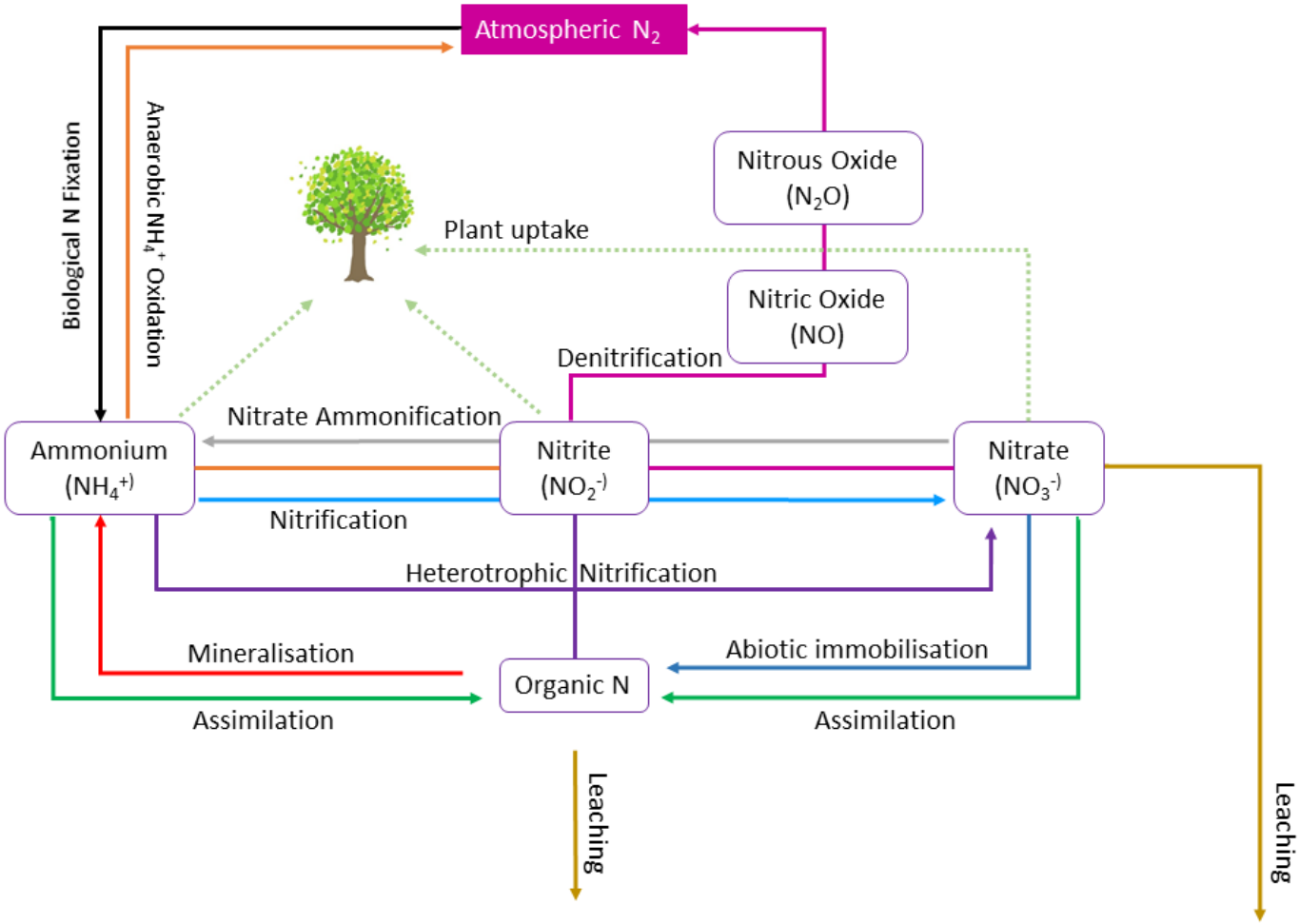


***Figure 1- 4:*** *Simplified schematic diagram of the terrestrial N cycle in a forest ecosystem*

### 1.4.2 Phosphorus Cycle

Phosphorus (P) has a less complex cycle than N regarding the various transformations. It is unique among the significant elemental biogeochemical cycles since it does not have a significant gaseous component. Soils' redox potential is too high to allow phosphine gas production (Bartlett, 1999), except in particular localised conditions (Dévai *et al.*, 1988).

Figure 1-5 shows a simplified schematic diagram of the terrestrial P cycle in a forest ecosystem. The availability of P in forests is sustained by P cycling and is determined by the competition between biological and geochemical sinks (Attiwill and Adams, 1993). Almost all P on the land is derived from chemical weathering, in which rainwater reacts with the parental material releasing soluble P ($PO_4^{3-}$) and other minerals ($Ca_3(PO4)_2$, $Mg_3(PO_4)_2$ and $FePO_4$). A part of the P in the soluble pool will be absorbed by the plant roots or immobilized by microbes. Through senescence or microbial population life cycle, the P returns to the soil in organic form, to be mineralized again to inorganic forms, or immobilized in the stable organic P pool in forms not available for biota.

Moreover, some of the soluble P can be geochemically transformed into secondary P minerals and enter the occluded P pool or undergo leaching. Young soils are abundant with P; however, P is very low with age, erosion, weathering, and leaching. Moreover, $PO_4^{3-}$ has a very slow diffusion rate in the soil solution and a fast sorption rate forming secondary minerals with Fe and Al under low pH (Parfitt, 1979). The solubility of these secondary minerals is very low, thus available P in the soil solution is minimal and often is less than 10 µM (Comerford, 1998).

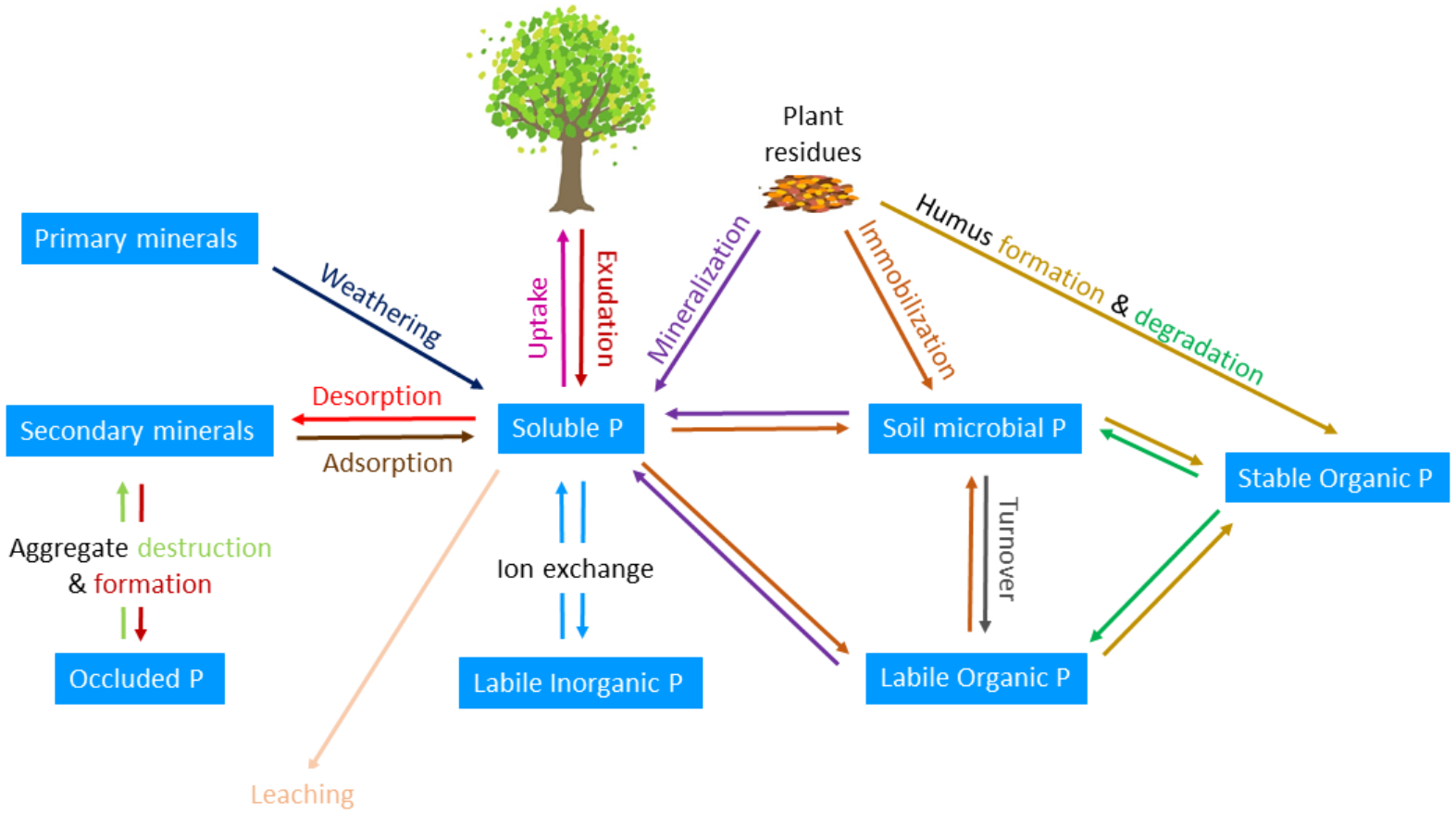


***Figure 1- 5:*** *Simplified conceptual model of the terrestrial P cycle in a forest ecosystem*

## 1.5 Future of Forests under a Changing Atmosphere

### 1.5.1 Atmospheric $CO_2$

For the past 800,000 years and up until the preindustrial era (~1750), the concentration of $CO_2$ in the atmosphere was approximately 280 ± 10 ppm. Ever since, it has risen at a fast pace, following global agricultural and industrial development, to a value of 407 ppm in 2018. This year (2019), the atmospheric concentration of $CO_2$ broke the record by exceeding 415 ppm (www.co2.earth, 2019), and it is predicted that the concentration will exceed 500 ppm by 2050 (Prentice *et al.*, 2001).

This increase is driven by the anthropogenic burning of fossil fuels, as evidenced by decreasing atmospheric $O_2$, increasing proportion of biogenic $^{12}C$-$CO_2$, and higher concentrations of atmospheric $CO_2$ in the northern hemisphere where most fossil fuel burning occurs (Prentice *et al.*, 2001). Nevertheless, atmospheric $CO_2$ increases only at about half the rate of fossil fuel emissions due to the ocean and terrestrial uptake currently acting as C sink.

$CO_2$, alongside methane ($CH_4$), nitrous oxide ($N_2O$), chlorofluorocarbons 11 and 12 (CFC-11 and CFC-12, respectively), and a group of another 15 minor gases are called greenhouse gases because they can absorb and gradually radiate heat. Although $CO_2$ absorbs less heat per molecule than $CH_4$ and $N_2O$, it has a longer lifetime in the atmosphere, can be found in larger quantities than the other greenhouse gases, and absorbs thermal energy from wavelengths $H_2O$ vapours are not able to (Lindsey, 2020).

One of the most crucial impacts of atmospheric $CO_2$ is the increase in Earth's temperatures, with land surface air temperature increasing faster than the global average temperature (by 1.53 and 0.87 °C, respectively), since the pre-industrial era (IPCC, 2019). Temperature increases have increased the occurrence, severity, and

extent of drought, heavy precipitation, and heat-associated events. As a result, climate change has induced growing season extension, browning of vegetation, shifts in climate zones, alterations to plant and animal species populations and life-cycles, sea-level rise and coastal erosion, permafrost thaw, threats to food security, variations in pests and diseases (IPCC, 2019). These effects are predicted to have different magnitudes globally; however, it is crucial to take mitigation actions since the fate of humans, biodiversity, and ecosystems is at risk.

It is expected that with the atmospheric $CO_2$ increase, plant processes and composition will be altered, thus affecting ecological communities (Hovenden and Williams, 2010). Increased atmospheric $CO_2$ will shift the C cycle and thus the other nutrient cycles, to which it is coupled, impacting the forest ecosystems and their function (Bonan, 2008). Subsequently, productivity and C storage will be affected, but the extent of the impacts is still not very well understood, and there is contradicting evidence in the literature. Thus it is crucial to understand how the elevated $CO_2$ will affect different ecosystems and gain more insight into the processes involved.

### 1.5.2 Impacts of elevated $CO_2$ on soil respiration

As described in section *1.1 Importance of forest ecosystems,* forest soils play a crucial role in uptaking atmospheric $CO_2$. Generally, elevated $CO_2$ increases the photosynthetic rate, thus the NPP (Norby *et al.*, 1999) and plant growth (Zak *et al.*, 2000). Moreover, several studies have shown increased rates of soil respiration and belowground C cycling enhancement (Janssens *et al.*, 1998; Lin *et al.*, 2001; Pataki *et al.*, 2003; Trueman and Gonzalez-Meler, 2005; Bernhardt *et al.*, 2006; Pregitzer *et al.*, 2006; Zhou, Wan and Luo, 2007; Deng *et al.*, 2010). Hence, although more C

might be sequestered in plant biomass under elevated $CO_2$ conditions, it might also be that a concurrent or greater amount is introduced to the atmosphere through respiration (Hungate *et al.*, 1997; Heath *et al.*, 2005; Trueman and Gonzalez-Meler, 2005), inhibiting the terrestrial ecosystems' capacity to sequester more C under increased atmospheric $CO_2$. Moreover, studies have shown that fine root biomass, the total number of roots and root length increased under elevated $CO_2$ (Rogers, Runion and Krupa, 1994; Matamala and Schlesinger, 2000; Tingey, Phillips and Johnson, 2000; Iversen, Ledford and Norby, 2008; Pritchard *et al.*, 2008), although root respiration *per se*, is not always affected (Matamala and Schlesinger, 2000), or not affected to a measurable degree (Edwards and Norby, 1999).

Furthermore, heterotrophic respiration is found to increase under elevated $CO_2$ due to increases in organic substrate available for decomposition from increased root production, turnover, and mortality, and litter inputs (Zak *et al.*, 2000; Deng *et al.*, 2010), or due to enhanced production and turnover of root exudates (Heath *et al.*, 2005). On the other hand, litter produced from plants grown under elevated $CO_2$ is found to have higher lignin and lower N concentrations, which may negatively impact heterotrophic respiration (Henry *et al.*, 2005; Knops, Naeem and Reich, 2007).

### 1.5.3 Impacts of elevated $CO_2$ on soil nutrients

Many studies have reported that elevated $CO_2$ is found to increase the NEP, with more substantial effects reported in higher latitudes, with temperate ecosystems being among them (Forkel *et al.*, 2016). Temperate ecosystems are also more N constrained since biological $N_2$ fixation is naturally low in these areas (Zaehle, 2013). The elevated $CO_2$ effects on soil N levels that have been reported are highly variable; they have

been shown to increase, decrease or remain stable (Gill *et al.*, 2002; Finzi and Schlesinger, 2003; Schneider *et al.*, 2004; Luo *et al.*, 2004; Reich *et al.*, 2006; Reich, Hungate and Luo, 2006; Dijkstra *et al.*, 2008; Langley *et al.*, 2009; Phillips, Bernhardt and Schlesinger, 2009; Norby *et al.*, 2010; Zak *et al.*, 2011; Mueller *et al.*, 2013; Rütting and Andresen, 2015).

The long term exposure to elevated $CO_2$ might result in limited N availability, thus inhibiting the $CO_2$ fertilization effect, and is referred to as the progressive N limitation hypothesis (Luo *et al.*, 2004; Dukes *et al.*, 2005; Hungate *et al.*, 2006; Reich, Hungate and Luo, 2006). Here, N becomes limited over time, sequestered into recalcitrant fractions of soil organic matter and long-lived plant tissues (Luo *et al.*, 2004). However, some studies suggest that progressive N limitation might not happen at all (Feng *et al.*, 2015) or maybe overcome (Lenka and Lal, 2012) since a lot of the recalcitrant biomass and soil organic matter pools have long turnover (<25 years), which is longer than most experiment's duration.

Little attention has been given to the effects of elevated $CO_2$ on P cycling or both the N and P dynamics. When P dynamics have been studied under field conditions, it was usually associated with N fertilization experiments. The results reported are conflicting since both increases (Khan *et al.*, 2010) or decreases (Lagomarsino *et al.*, 2008), depending on N fertilization levels. Hasegawa et al., (2016) reported increased N and P availability in a P-limited mature Eucalyptus forest (EucFACE), under elevated $CO_2$, due to increased plant investment belowground on labile C and the associated increase of the microbial turnover of organic matter and mobilization of chemically non-available P.

### 1.5.4 Models uncertainty

Future changes in terrestrial carbon uptake will affect atmospheric $CO_2$ concentrations and, thus, the climate. Therefore the use of models for future predictions is essential. Several studies have shown that the biosphere operates as a net C sink, offsetting the climate impact of fossil fuel burning and deforestation, until positive responses from climate change, such as decreased productivity, increased soil respiration or dieback (Cox *et al.*, 2013), and land-use change emissions outpace the $CO_2$ effect. The extent of this feedback is critical to simulating future climate, and the results reported from observations are contradictory; the uncertainty of the land's C uptake capacity remains high (Friedlingstein *et al.*, 2006; Arora *et al.*, 2013; Jones, Stott and Christidis, 2013; Lovenduski and Bonan, 2017).

## 1.6 FACE experiments

The effects of elevated $CO_2$ in plants have been well studied in individual species grown in controlled environments or enclosures (Kimball, 1983; Ceulemans and Mousseau, 1994; Gunderson and Wullschleger, 1994; Amthor, 1995; Curtis, 1996; Drake, Gonzalez-Meler and Long, 1997; Curtis and Wang, 1998; Saxe, Ellsworth and Heath, 1998; Norby *et al.*, 1999; Wand *et al.*, 1999). However, these studies faced potential severe limitations due to the enclosed systems, such as restricted root growth or amplified photosynthetic and production rate due to 'chamber effect' (Arp, 1991; McLeod and Long, 1999; Morgan *et al.*, 2001). A solution to the confinements of controlled environment limitations was the free-air $CO_2$ enrichment (FACE) experiments in the 1990s (Hendrey, Lewin and Nagy, 1993; Lewin *et al.*, 1994; Miglietta *et al.*, 2001; Okada *et al.*, 2001). These experiments allowed plant exposure

to elevated $CO_2$ under natural conditions with no enclosed infrastructure, with temporal and spatial $CO_2$ concentration control throughout the canopy levels.

FACE experiments have been established in various ecosystems worldwide, studying the effects of elevated $CO_2$ in different species and climatic conditions (Table 1-1). FACE technology has proven to be an advantageous method providing understanding and data on the responses of the ecosystem as a whole, over spatial and temporal scales, depicting a future ecosystem image, as accurate as possible (Nowak, Ellsworth and Smith, 2004; Ainsworth and Long, 2005; Norby *et al.*, 2005; Hyvönen *et al.*, 2007; Norby and Zak, 2011).

The FACE experiments conducted from the mid-90s to mid-2000s in temperate forested ecosystems (Table 1-1) offered new insights into the forest responses under a step increase of atmospheric $CO_2$, C allocation, nutrient interactions, stand development (Norby and Zak, 2011), and critical improvements to models (Medlyn *et al.*, 2015). However, these experiments were conducted in young, relative homogenous stands on agricultural or disturbed soils, thus limiting the understanding of the elevated $CO_2$ effect to the ecosystem as an entity, the C cycle and allocation in a mature forest, and the interactions among different plant species (Norby *et al.*, 2016), Hence, FACE experiments were needed in mature ecosystems since young and mature forests sequester different amounts of C and have different C and nutrient needs. EucFACE and Web-FACE are the only two forest FACE experiments conducted in mature forests (mixed temperate and Eucalyptus forests, respectively). Web-FACE was conducted in specific stands rather than using the “ring” approach that all other FACE experiments used.

***Table 1- 1:*** *Forest Free Air $CO_2$ enrichment (FACE) experiments in a descending chronological order*

| Site | Location | Ecosystem | Ecosystem age (years) | Elevated $CO_2$ (ppm) | First-year of exposure | Status |
|---|---|---|---|---|---|---|
| EucFACE | Richmond, Australia | Cumberland Plain forest | >75 | 550 | 2012 | Ongoing |
| Web-FACE | Hofstetten, Switzerland | Mature temperate forest | 80-120 | 600 | 2000 | Closed |
| POPFACE | Viterbo, Italy | Poplar plantation | Planted cuttings | 550 | 1999 | Closed |
| ASPEN FACE – FACTS II | Rhinelander, Wisconsin, USA | Aspen forest | Planted | Ambient + 200 | 1998 | Closed |
| ORNL FACE | Oak Ridge, Tennessee, USA | Sweetgum plantation | Planted | Ambient+200 | 1998 | Closed |
| Duke FACE – FACTS I | Duke Forest, North Carolina, USA | Loblolly pine forest | ~16 | Ambient + 200 | 1996 | Closed |

## 1.7 BIFoR FACE experiment

Although the FACE experiments that happened in the 1990s-2000s offered crucial ground for testing assumptions and responses of forested ecosystems under elevated $CO_2$, they were conducted in young, homogenous forested areas or recovering from disturbance ecosystems. Birmingham's Institute of Forest Research (BIFoR) FACE facility is the only FACE experiment in a mature temperate forest in the northern hemisphere, and alongside EucFACE in the southern hemisphere, are the only two 'second generation' FACE experiments in the world, testing the responses of undisturbed old-growth forests under elevated $CO_2$.

Mature forests are expected to respond differently to elevated $CO_2$ than young forests due to different developmental C needs and different internal-plant C allocations (Körner, 2006). Moreover, old-growth trees may have fully explored the belowground resource space, limiting the possibility of increased C allocation belowground to increase access to limiting nutrients (Körner, 2006). Even if mature trees can allocate more C belowground, this does not necessarily mean that the nutrient uptake will be higher if the belowground resources are already explored. Furthermore, because nutrient cycles in mature forests are more coupled than young forests, N losses might be limited in old-growth ecosystems (Zaehle *et al.*, 2014). Besides, mature trees have exhibited less flexibility in their C and nutrient use (Körner *et al.*, 2005), thus less likely to increase whole-plant nutrient use efficiency (Bader *et al.*, 2013).

BIFoR FACE facility was established in 2015 in an old-growth (>160 years) temperate forest in Staffordshire (UK) and currently is in the third year of fumigation (Fig. 1-6). Over ten years, BIFoR aims to investigate the impacts of elevated $CO_2$ in a mature temperate forest, C uptake and storage, nutrient dynamics and water use efficiency,

ecosystem structure and function, biodiversity, and susceptibility to abiotic and biotic stress.

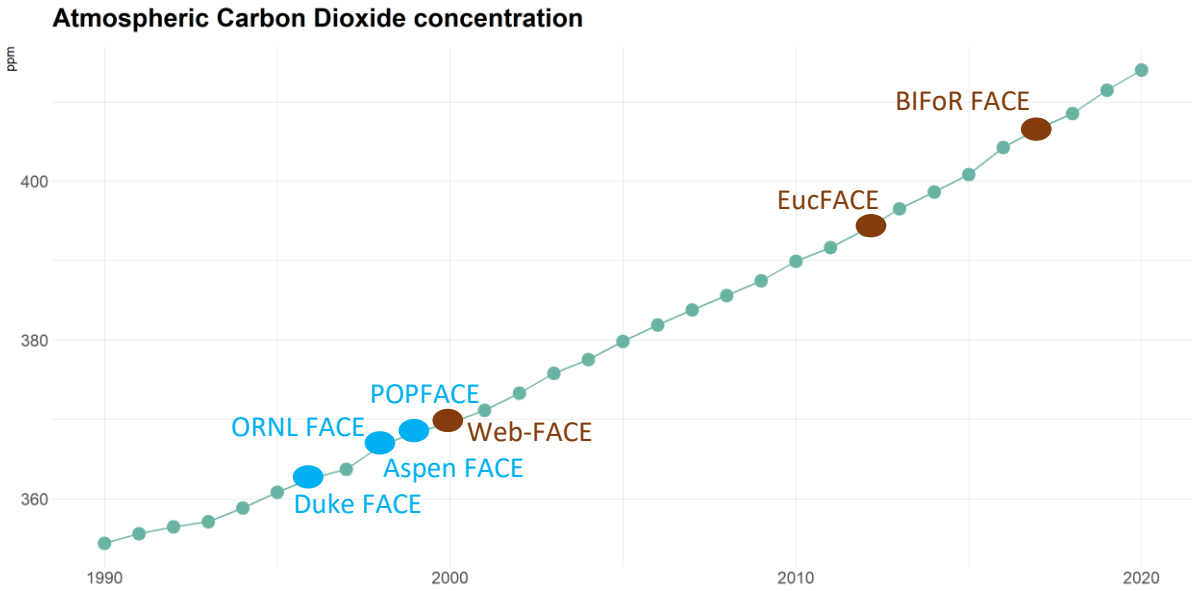


***Figure 1- 6:*** *Previous forest FACE experiments and the atmospheric $CO_2$ concentration during their first year of $eCO_2$ enrichment. With light blue colour are the FACE experiments conducted in young forests of plantations, while with dark red colour are the FACE experiments conducted in mature forests. Note that only EucFACE and BIFoR FACE are still in operation (graph adapted by David Ellsworth presentation, atmospheric $CO_2$ concentration data from* Tiseo, 2021).

## 1.8 Thesis layout

This thesis aims to assess the response of forest soil biogeochemical cycles (C, N, P) to elevated $CO_2$; to monitor soil respiration, nutrient bioavailability, and microbial biomass under ambient and $eCO_2$ atmosphere at BIFoR FACE. The soil respiration is partitioned to root, hyphal and microbial respiration to quantify each soil component's contribution to soil respiration and its response to elevated $CO_2$. Bioavailable soil N and P dynamics are assessed using the ion-exchange resin membranes technique, while microbial biomass assessments are achieved periodically. This thesis utilises the techniques mentioned above to the highest resolution up to date in such a large-

scale climatic experiment. Hence this dataset's uniqueness makes it the first of its kind with a paramount resolution both spatially and temporally.

Chapter 2 provides a detailed outline of both the experimental designs and methodological approaches in this thesis (for *Chapters 3, 4, and 5*). It includes both field and laboratory techniques and elaborates on the statistical avenues followed for the thesis, which can be found in detail in the respective Chapters.

Chapter 3 evaluates the belowground responses before any $eCO_2$ enrichment at BIFoR FACE and during the first year of operation. Specifically, the short term responses of soil respiration and nutrient dynamics were monitored approximately 6 months before the $eCO_2$ enrichment until the end of the first year of $eCO_2$ enrichment.

Chapter 4 investigates the belowground responses during the second year of $eCO_2$ enrichment at BIFoR FACE, which coincided with a series of extreme events (Beast from the East, winter moth outbreak, heatwave). Soil respiration, nutrient dynamics and microbial biomass responses were monitored under the combined effects of $eCO_2$ enrichment and extreme events.

Chapter 5 quantifies the soil respiration partition to autotrophic and heterotrophic, as well as the further autotrophic partitioning to root and mycorrhizal respiratory components during the first two years of $eCO_2$ enrichment at BIFoR FACE.

Chapter 6 is a synthesis of the scientific outcomes of Chapters 3, 4, and 5, while Chapter 7 is a future outlook perspective on the potential further research that can improve the current understanding of belowground responses under $eCO_2$.

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# Chapter 2: Methodology

This chapter gives an overview of the site selected for study and the field and the analytical methods used to produce the results set out in Chapters 3 to 5 and aims to provide general information about the study area and the methodological approaches used. Detailed descriptions about the site and the FACE Facility can be found at Hart *et al.*, (2019) and Mackenzie et al., (2021).

## 2.1 Site Description

### 2.1.1 Birmingham's Institute of Forest Research (BIFoR)

Birmingham's Institute of Forest Research (BIFoR) is temperate deciduous woodland, located within Mill Haft at Staffordshire, England (52º 48' 3.6" N, 2º 18' 0" W). Mill Haft is part of Norbury Park Estate and has been woodland since pre-1881, appearing to be unchanged ever since. The total BIFoR FACE experimental area covers 7.3 ha, while the great forest covers 19.1 ha (Figure 2-1). A detailed site description can be found in Mackenzie *et al.* (2021).

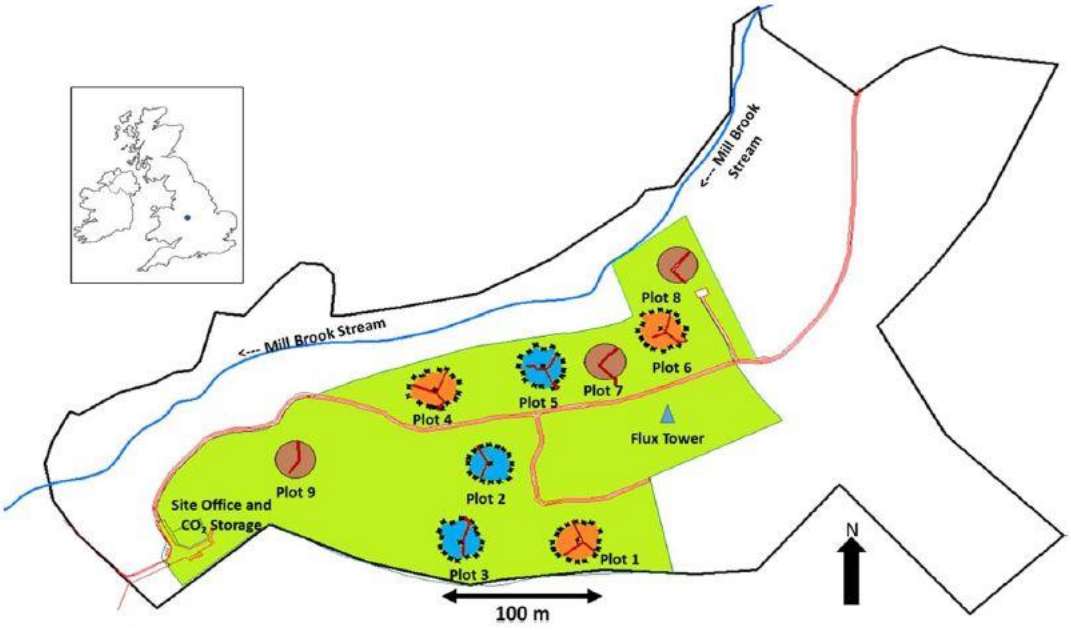


***Figure 2- 1:*** *Schematic map of the BIFoR FACE facility with Mill Haft wood. The green coloured area highlights the total experimental area controlled by the University of Birmingham, while the thick black line area represents the greater forest area. The circles coloured in orange, blue, and brown represent the eCO2, ambient, and ghost arrays, respectively. The red line represents the main access road, while the blue line represents the Mill Brook stream flowing through the northern side of the woodland from NNE to WSW. The blue triangle represents a 40 m flux tower (adapted from Hart et al., 2019).*

BIFoR has a temperate maritime climate, with a mean annual temperature of 8 °C and mean annual precipitation of 800 mm (Mackenzie et al., 2021). Briefly, the soil is brown earth luvisol with a sandy-clay texture and a mean soil pH of 4.5 at the top 10 cm. Mean soil total N and P in the top 10 cm are 0.28% and 18 mg P $kg^{-1}$, respectively, indicating that BIFoR is a low nutrient woodland, and total organic matter content is 3.67%.

The dominant overstorey species at the BIFoR FACE facility is old-growth (>160 years) *Quercus robur* (pedunculate oak), approximately 25 m height, covering approximately 45% of the array area. The woodland has a dense multi-layered canopy unaffected by the construction of the FACE infrastructure (Hart *et al.*, 2019). The understorey is interspersed with *Corylus avellana* (common hazel), *Acer pseudoplatanus* (sycamore), *Ilex aquifolium* (holly), *Crataegus monogyna,* and *C.*

*laevigata* (hawthorn). Pre-treatment oak leaf analysis showed that the mean N:P ratio was ~16, indicating a possible N and P co-limitation in the ecosystem (Mackenzie et al., 2021).

### 2.1.2 BIFoR Free Air $CO_2$ Enrichment (FACE) facility

A detailed FACE facility description can be found in Hart *et al.* (2019). Briefly, the BIFoR FACE facility comprises six 30 m diameter infrastructure arrays (elevated $CO_2$, n = 3; ambient, n = 3) and three non-infrastructure arrays (undisturbed, n = 3). Each treatment is replicated three times and operates on two different canopy levels, as suggested by Filion, Dutilleul, and Potvin (2000). The infrastructure is approximately 25 m height, depending on each array's canopy height. Figure 2-2 depicts a schematic example of a BIFoR FACE array with infrastructure. $CO_2$ is mixed with air before its release and subsequently is released from the upwind quadrant of 15-m radius treatment patches. The $CO_2$ release rates are adjusted every second, and the emitter pipe valve, which is based on wind direction, is adjusted every 4 seconds.

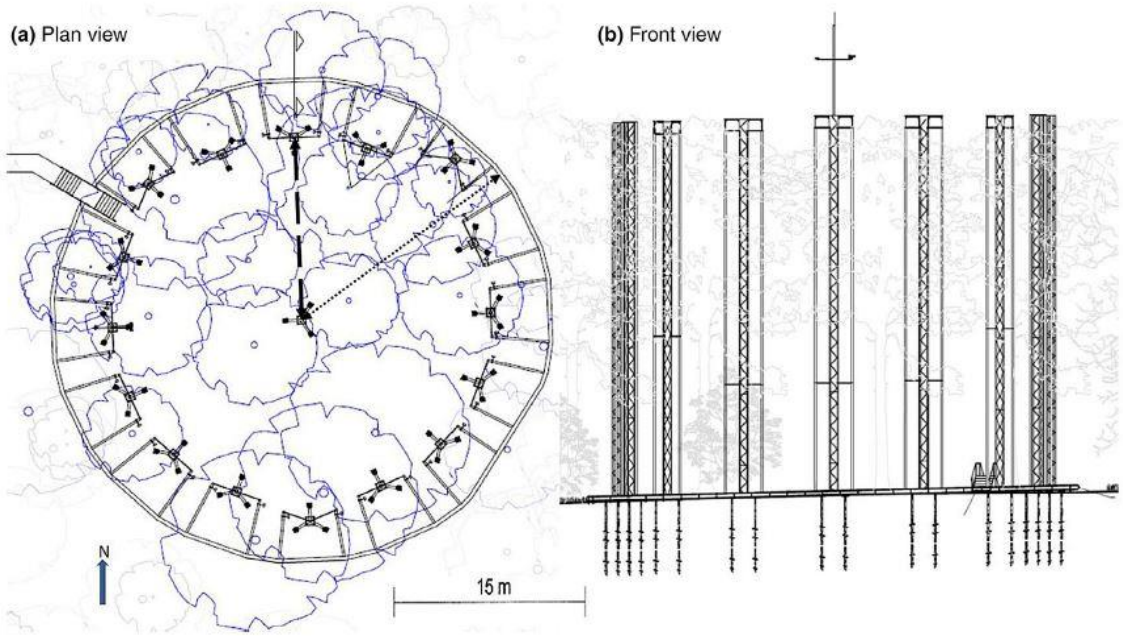


***Figure 2- 2:*** Schematic representation of a BIFoR FACE infrastructure array. (a) Plan view of a 30 m diameter experimental patch with the structures at its circumference. The dotted line represents the center's radius to the outer plenum edge (R ~ 20 m). In contrast, the dashed line represents the radius from the centre to the research array's inner edge (R ~ 15 m). (b) The front view depicts the screw pile system penetrating the bedrock, providing secure anchoring and bypassing the need for supporting the towers with guy cables (Hart *et al.*, 2019).

$CO_2$ fumigation began on the 3rd April 2017 until 27th October 2017 (Year 1) and on the 1st April 2018 until 31st October 2018 (Year 2). Both years, the beginning and the end of the fumigation followed the forest's phenology, coexisting with the beginning of budburst and leaf fall, respectively. The fumigation system operated from 05:00 to 22:00, depending on the solar angle. For Year 1 (Figure 2-3), the $eCO_2$ arrays were within the 10% of target for 81.6% of the scheduled operation time and 20% of the target within 96.7% (Hart *et al.*, 2019). Contamination of the ambient arrays by $CO_2$ from the $eCO_2$ arrays was rare and short-lived, with ambient arrays being 10% of the control setpoint 98.8% of the time.

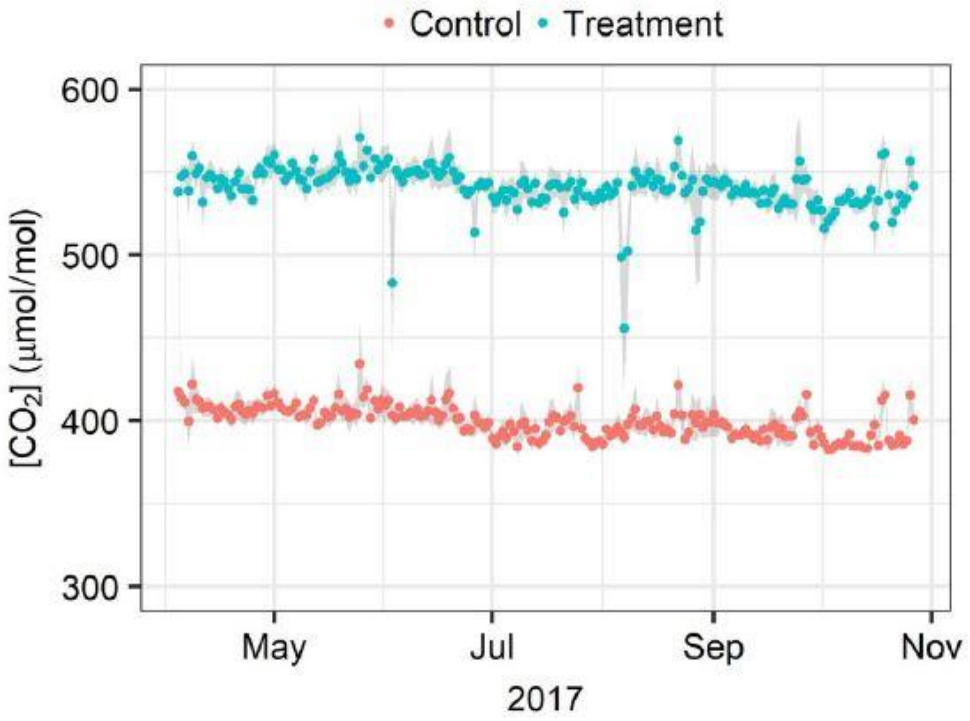


***Figure 2- 3:*** *Daily atmospheric $CO_2$ averages in ambient (red dots) and eCO2 (blue dots) arrays at BIFoR FACE during Season 1 (4th April – 27th October 2017) (adapted from Hart et al., 2019).*

## 2.2 Field Methods

## 2.2.1 Soil respiration

### 2.2.1.1 Experimental design

To assess the soil respiration at BIFoR FACE, 18 experimental blocks were established across the site, 9 in ambient arrays and 9 in $eCO_2$ arrays (Figure 2-4), as proposed by Heinemeyer *et al.* (2007). Collars were installed in June 2016 (9 months before $CO_2$ fertilization), minimizing soil disturbance on soil respiration measurements, and left during the summer period for settlement.

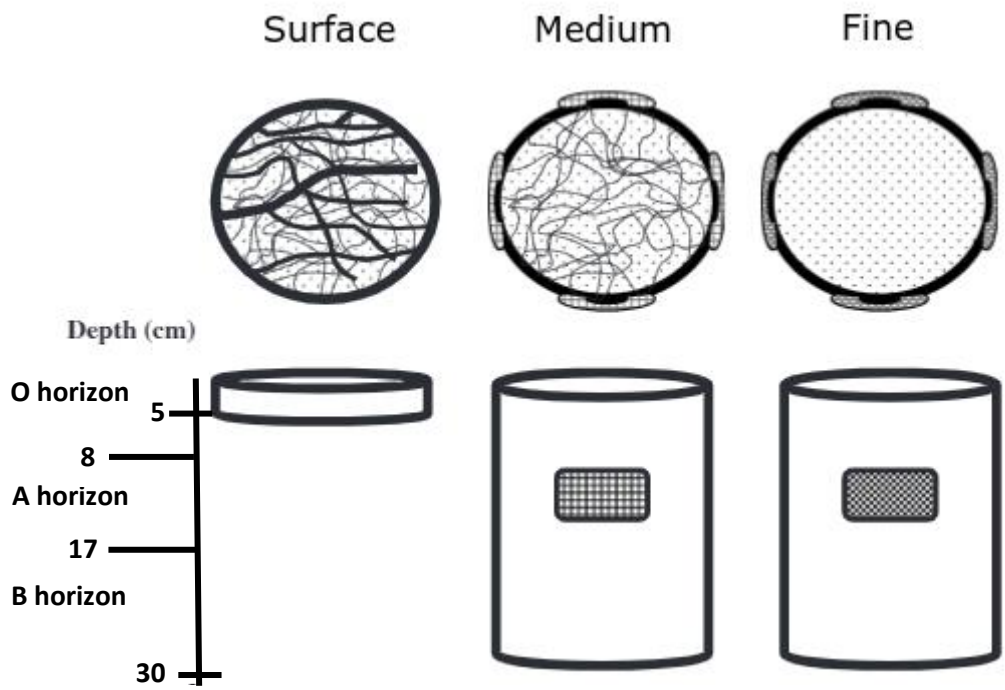


***Figure 2- 4:*** *Schematic representation of the soil respiration experimental blocks used at BIFoR FACE (adapted from Heinemeyer et al., 2007). Surface collars were only 5 cm deep inserted in the soil allowing roots, hyphal, and microbial growth. Medium and fine collars were inserted 30 cm deep in the soil, excluding fine roots from entering the collar. The fine collars were allowing only microbial growth.*

Each block consisted of one PVC shallow collar (Drainage Superstore, Plymouth, UK) and two deeper collars with different mesh treatments. All collars were 200 mm in diameter, the shallow collars (*surface* collars hereafter) were 5 cm in height, and the deeper ones were 30 cm in height. The shallow collar was inserted only 3 cm in the soil, pressed firmly onto the litter layer with three 20 cm long stainless steel rods attached at the bottom of the collar to prevent dislocation by human or animal activity. The stainless steel rods ensured no roots were severed while an airtight seal was achieved, avoiding potential lateral diffusion of $CO_2$. These collars did not interfere with fine roots, mycorrhizal, or microbial dynamics and growth. The two deeper collars were inserted at approximately 25 cm depth, preventing fine root ingrowth, which is concentrated mainly in the soil's upper centimetres (Jackson, Mooney, and Schulze, 1997). Deeper collars had four rectangular (5 x 5 cm) windows cut into their sides

which were covered with nylon meshes, with grids size of 41 µm (Ek, 1997) (*medium* collars hereafter) and 1 µm (*fine* collars hereafter), allowing or excluding ectomycorrhizal hyphal ingrowth, respectively. In summary, there are three surface, three medium, and three fine collars in every ambient and $eCO_2$ array (18 collars of each treatment across the site).

As mentioned in Chapter 1, assessing the contribution of various soil components to the total soil respiration is of paramount importance towards understanding the C dynamics belowground and their possible imbalance due to $CO_2$ fertilization. With the experimental design as described above, partitioning the soil respiration into total ($R_s$), autotrophic ($R_a$), heterotrophic ($R_h$), fine root ($R_r$), and hyphal ($R_m$) respiration were enabled. $R_s$ and $R_h$ were measured directly from the surface and fine collars, respectively. $R_a$ was calculated by subtracting the fine collars' values from those of the surface collars. Rr was calculated by subtracting the medium collars' values from the surface collars, and lastly, $R_m$ was calculated by subtracting the fine collars' values from the medium collars. For enabling a better understanding, the equations used for the soil respiration partitioning calculations can be found below:

$$Total\ soil\ respiration\ (R_s) = \ Surface\ collars \qquad Equation\ 2-1$$

$$Heterotrophic\ respiration\ (R_h) = \ Fine\ collars \qquad Equation\ 2-2$$

$$Autotrophic\ respiration\ (R_a) = \ Surface - \ Fine\ collars \qquad Equation\ 2-3$$

$$Fine\ root\ respiration\ (R_r) = \ Surface - \ Medium\ collars \qquad Equation\ 2-4$$

$$Hyphal\ respiration\ (R_m) = \ Medium - \ Fine\ collars \qquad Equation\ 2-5$$

#### 2.2.1.2 Soil respiration measurements

Soil respiration rates were measured with a close-path infrared gas analyser (IRGA) control unit (LI-8100A, LI-COR, Nebraska, USA) coupled with a multiplexer (LI-8150, LI-COR, Nebraska, USA), allowing all nine collars to be measured using opaque long-term chambers (8100-104, LI-COR, Nebraska, USA), autonomously and continuously for one ambient and one eCO2 array simultaneously. The first measurement took place in October 2016 (4 months after the collar installation) and continued until October 2018 (end of Year 2 of the $CO_2$ fumigation). The measurements were collected on hourly intervals for two continuous weeks, allowing the monitoring of soil respiration variability associated with daily cycles. The instruments were moved to the next ambient and $eCO_2$ arrays on a fortnightly basis, enabling the monitoring of soil respiration associated with spatial variability.

$CO_2$ travels from the production sites to the atmosphere principally by diffusion through air-filled pores and cracks in the soil. However, it can also be impelled by local climatological changes in pressure due to wind or volumetric displacement by rain. The LI-8100A avails the rate of $CO_2$ increase in the chamber to estimate the rate at which $CO_2$ diffuses into the air outside the chamber; thus, for accurate estimates, conditions in and out of the chamber must be similar. These conditions contain the concentration gradients driving diffusion, barometric pressure, soil moisture, and temperature.

As the chamber closes to measure, it creates an increase in $CO_2$ mole fraction, making the $CO_2$ diffusion gradient between the soil surface layer and the air inside and outside of the chamber differ. The instrument estimates and corrects the diffusion rate using an analytical technique that considers the effects of increasing chamber $CO_2$

concentration on the diffusion gradient, facilitating the initial rate of $CO_2$ increase estimate that followed immediately after the chamber closed.

Although previous studies have shown that chambers can change gas concentration gradients in the soil, causing errors in $CO_2$ flux estimates (Healy *et al.*, 1996), this is prevented by limiting the measurement time to 2 minutes, limiting $CO_2$ concentration changes to vary significantly, and keeping the chamber effect to the minimum. Furthermore, chambers are designed with pressure vents to block pressure gradients and wind incursions outside of the chamber. The chamber's automated movement prevents possible mechanical disturbances during measurements. The soil area inside and outside of the chamber is exposed to the same environmental conditions, as the chamber moves away from the soil measurement area when a measurement is not in progress.

The instrument automatically provides the calculation of fluxes and performs the essential corrections by default. A detailed description of the system's theory of operation can be found at *LI-8100 Automated Soil CO2 Flux System & LI-8150 Multiplexer Instruction Manual* (2007). Briefly, the equation used for calculating the flux is:

$$F_c = \frac{10 * V * P_0 \left(1 - \frac{W_0}{1000}\right)}{R * S(T_0 + 273.15)} * \frac{\partial C'}{\partial t} \qquad Equation\ 2-6$$

Where $F_c$ is the soil $CO_2$ efflux rate (µmol $m^{-2}$ $s^{-1}$), V is the volume ($cm^3$), $P_0$ is the initial pressure (kPa), $W_0$ is the initial water vapour mole fraction (mmol $mol^{-1}$), S is the soil surface area ($cm^2$), R is the gas constant (8.314 Pa $m^3$ ($K^{-1}$ $mol^{-1}$)), $T_0$ is the initial

air temperature (°C), and $(\partial C')/\partial t$ is the initial rate of change in water-corrected $CO_2$ mole fraction (µmol $mol^{-1}$).

Data quality was assessed using the SoilFluxPro™ software (LI-COR, Nebraska, USA). Since the infrared gas analyser-multiplexer-long-term-chamber path is closed, negative fluxes were removed from the dataset. Furthermore, fluxes with a coefficient of variation (%) of linear flux greater than 3.5, and fluxes with poor fitting were also removed. The software calculates both the linear and the exponential soil flux automatically; however, certain variables were adjusted from the manufacturer's default settings to fit our experimental needs. The surface area of the 20 cm diameter collars was 314.16 $cm^2$, the long-term chamber volume was 4076.1 $cm^3$, while the IRGA and the multiplexer volume were set to default values. The volume of the cables (237 $cm^3$) connecting the chambers with the multiplexer was also included. Due to soil variability, each chamber-collar surface's overall volume was unique for each collar and was measured frequently to ensure accurate fluxes, as advised by the manufacturer. All observations were 2 minutes in length, with the first 20 sec excluded from the flux calculation since it is a period that ensures the gas mixing inside the chamber. An additional 15 sec and 45 sec were added before and after the observation duration, respectively, to ensure that the cables transferring the gas to the IRGA were flushed clean and avoid any potential carryover to the following observation.

### 2.2.2. Environmental factors

Soil volumetric water content (VWC) and soil temperature ($T_s$) were measured one meter from each of the three experimental soil respiration blocks per array, with

permanently installed shallow CS655 probes inserted in the top 12 cm soil (Campbell Scientific, Logan, UT, USA). These data were recorded in 15-min intervals by a data logger in each plot (Campbell Sci CR1000, Campbell Scientific).

### 2.2.3 Soil Bioavailable nutrients

Ion-exchange resin membranes (IEMs hereafter) (Membranes International Inc., New Jersey, USA) were used to assess inorganic N, as ammonium ($NH_4^+$) and nitrate ($NO_3^-$), and P, as phosphate ($PO_4^{3-}$), availability, using a similar approach as Hasegawa, Macdonald and Power (2016). This is a simple, commonly used method offering an integrated measure of nutrient availability through the whole incubation period (Abrams and Jarrell, 1992; Qian and Schoenau, 2002; Bowatte *et al.*, 2008) as opposed to nutrient availability measurements from soil extracts and soil solutions that provide instantaneous measurements of nutrient availability, echoing the concentrations available at the time of the sampling (Abrams and Jarrell, 1992; Bowatte *et al.*, 2008).

Five anion IEMs (AMI 7001; 2 cm x 12 cm) and five cation IEMs (CMI 7000; 2 cm x 12 cm) were inserted in the top 12 cm of the soil next to each soil respiration experimental block in each array. From January 2017, three AMI and three CMI were inserted in the soil at eight locations within each array; three sets of each were located next to the soil respiration experimental blocks. The rest five sets were randomly located within each array. IEMs were incubated in situ for approximately a month before retrieval.

## 2.2.4 Soil sampling

### 2.2.4.1 Experimental design

Samples were collected in March 2017 (a month before the $CO_2$ treatment started) and then in March, July, and November 2018 (Year 2). No samples were collected during Year 1 of the experiment due to logistical constraints such as time, resources, and human power availability. Before the commencement of soil sampling, three permanent soil plots (2 x 2 $m^2$) for soil core extraction have been marked out within each array ($eCO_2$, ambient, and ghost). The soil plot locations are specified in Fig. 2-5. Each soil plot was subsequently subdivided into a grid with a plastic frame representing the available core extraction area. For each soil plot, a corresponding plot outside each array was marked for backfilling core extraction.

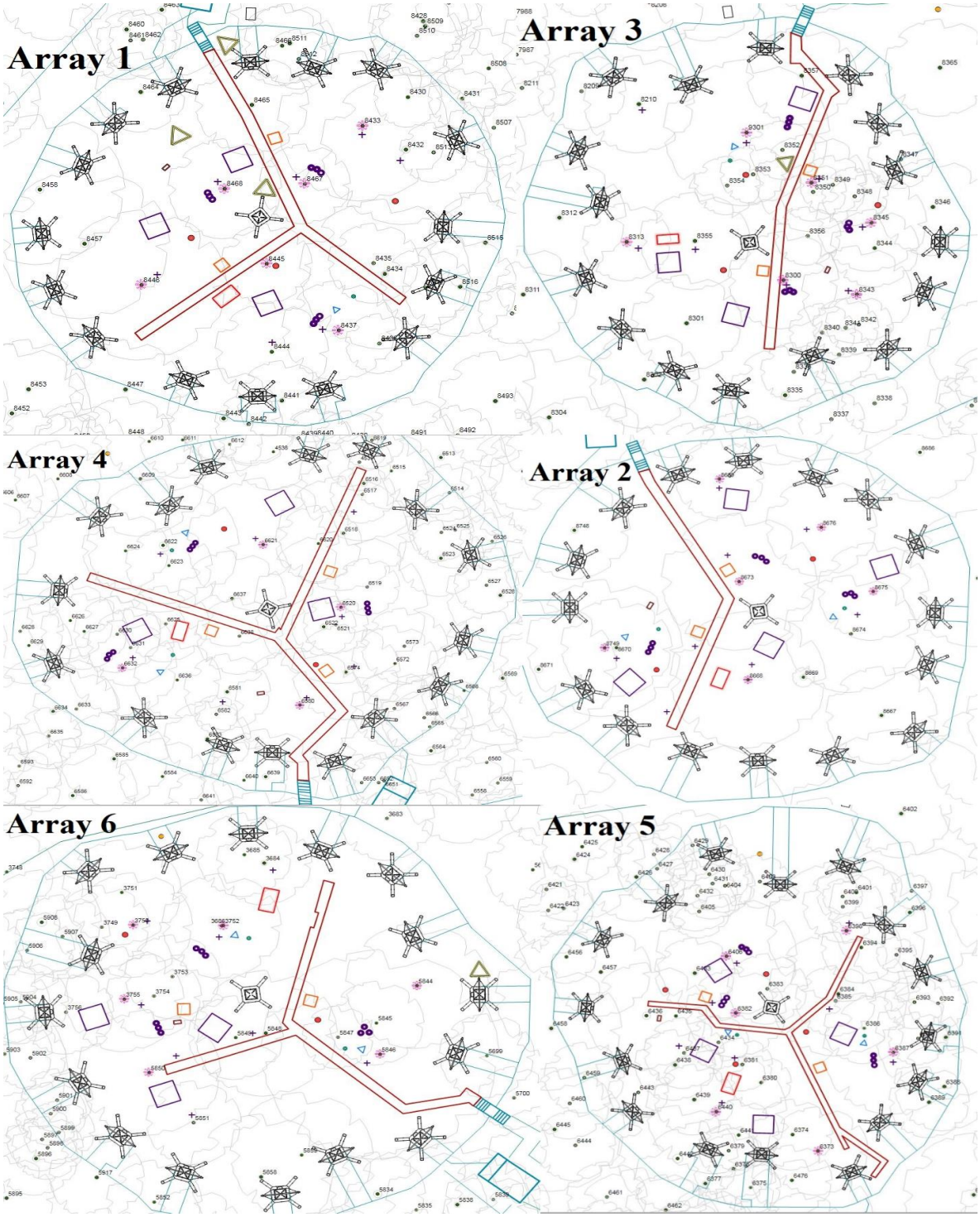


***Figure 2- 5:*** *Maps of all six FACE arrays (n = 3 ambient and n = 3 $eCO_2$) shown as the initially assigned pairs (ambient-$eCO_2$). The purple squares denote the soil sampling plots (n = 3 per array, except array 2 and 5 where n = 4), the purple circles denote the soil respiration experimental blocks (n = 3 per array), and the purple crosses denote the ion-exchange soil resin membrane locations (n = 8 per array).*

### 2.2.4.2 Soil core extraction

A 55 mm-diameter soil core (30 cm depth) was extracted from each soil plot from each of the nine arrays (n = 27 soil cores across the site). All soil cores were sampled on the same day and kept in a cool box as generally advised for temperate soils (Hart *et al.*, 1994) and widely used when there are constraints on possessing soil samples directly after sampling. For each soil core taken from the soil plots in each array, the same depth and diameter core was taken from the backfilling plots outside of the arrays. The backfilling cores were excavated to the same depth as the soil cores, separated by horizons, and sieved with a 2 mm mesh sieve. Roots and pebbles were removed from the backfilling cores. Both locations from where soil and backfilling cores were extracted were marked with a 20 cm long plastic tube. A summary of the samples taken over the four different sampling campaigns is set out in Table 2-1.

***Table 2- 1:*** *Summary of the number of samples taken, analysis, and methods used for all four soil campaigns at BIFoR FACE.*

| | | n (per sampling campaign) | | | |
|---|---|---|---|---|---|
| | | Baseline | Season 2 | | |
| Variable | Method | March (2017) | March (2018) | July (2018) | November (2018) |
| Soil moisture | Loss of ignition | 81 | 81 | 81 | 81 |
| Bulk density | 55 mm diameter x 30 cm length core | 27 | 27 | 27 | 27 |
| Microbial biomass | Chloroform fumigation | 81 | 81 | 81 | 81 |
| Total organic C | Analytik Jena | 162 | 162 | 162 | 162 |
| Total organic N | Analytik Jena | 162 | 162 | 162 | 162 |
| $NH_4^+$ | Skalar San ++ | 81 | 81 | 81 | 81 |
| $NO_3^-$ | Skalar San ++ | 81 | 81 | 81 | 81 |
| $PO_4^{3-}$ | Molybdenum blue method | 162 | 162 | 162 | 162 |

## 2.3 Laboratory Methods

### 2.3.1 Soil Bioavailable nutrients

Upon retrieval, IEMs were washed thoroughly with deionized water to remove any soil particles and then extracted for $PO_4^{3-}$, $NH_4^+$ and $NO_3^-$ using the following modification of Rayment and Lyons (2011)'s and Bowatte *et al.* (2008)'s methods, respectively. Each AMI and CMI from each sample location were extracted with 0.5 M HCl, shaken at 180 rpm for two hours, and then the membranes were removed from the solution and discarded. From January 2017 and onwards, the three CMI from each location were bulked together as one sample, and equivalently for the three AMI, and then the same process was followed as described before.

Concentrations of $NH_4^+$ and $NO_3^-$ were analysed by a continuous flow analyser (San ++ Continuous Flow Analyser, Skalar, Breda, The Netherlands). The automated procedure for $NH_4^+$ determination is based on the modified Berthelot reaction (Krom, 1980; Searle, 1984). Ammonia is chlorinated to monochloramine, which reacts with salicylate to 5-aminosalicylate. After oxidation and oxidative coupling, a green coloured complex is formed. The absorption of the formed complex is measured at 660 nm. Six standards were used for performing the calibration curve, reference material was used every 30 samples, and a drift check material was used every ten samples to assess potential variation over the analysis time. The software performs drift and water correction automatically.

Moreover, the automated procedure for the determination of $NO_3^-$ is based on the cadmium reduction method (Navone, 1964; Gal, Frenzel, and Möller, 2004). The sample is buffered at pH 8.2 and passed through a column containing granulated copper-cadmium to reduce the $NO_3^-$ to nitrite ($NO_2^-$). The $NO_2^-$ (originally present plus reduced $NO_3^-$) is determined by diazotising with sulphanilamide and coupling with N-

(1-naphthyl)ethylenediamine dihydrochloride to form a highly coloured azo dye which is measured at 530 nm. Six standards were used for performing the calibration curve, reference material was used every 30 samples, and a drift check material was used every ten samples to assess potential variation over the analysis time. The software performs drift and water correction automatically.

Concentrations of $PO_4^{3-}$ were analysed spectroscopically (UV-Vis Jenway 6850, Cole-Palmer, UK) using the molybdenum blue method (Boltz and Mellon, 1948; Worsfold *et al.*, 2005). Ammonium heptamolybdate and potassium antimony (III) oxide tartrate react in an acidic medium with diluted phosphate solutions to form an antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-coloured complex by L(+)ascorbic acid and measured at 880 nm. Six standards were used for performing the calibration curve, reference material was used every 30 samples, and a drift check material was used every ten samples to assess potential variation over the analysis time. Standards and samples were prepared in 4 mL cuvettes and were left for 15 minutes for colour development.

Detection limits for all three analyses can be found in Table 2-2 as provided by Skalar and as calculated via the l method (Shrivastava and Gupta, 2011). The biota-available N and P ratios were determined as ratios of $NH_4^+$-N, $NO_3^-$-N, and $PO_4^{3-}$-P. The data presented in this study are reported in monthly averages in µg $NH_4^+$-N $cm^{-2}$ $d^{-1}$, µg $NO_3^-$-N $cm^{-2}$ $d^{-1,}$ and µg $PO_4^{3-}$-P $cm^{-2}$ $d^{-1}$, respectively.

***Table 2- 2:*** *Summary of detection limits and wavelengths used during the soil bioavailable nutrient analysis, inorganic N, and microbial P analyses provided by the manufacturer (for $NH_4^+$ and $NO_3^-$) and as calculated (for $PO_4^{3-}$). The units reported are mg $NH_4^+$-N, $NO_3^-$-N, and $PO_4^{3-}$-P /L. Detection limits for all three analyses were calculated using 0.5M HCl as the matrix.*

| | | Detection limits | | | |
|---|---|---|---|---|---|
| Analysis | Wavelength | Instrument's specification | Limit of detection | Limit of quantitation | Limit of blank |
| $NH_4^+$ | 660 nm | 0.2 | 0.143 | 0.426 | 0.089 |
| $NO_3^-$ | 530 nm | 0.02 | -0.041 | 0.015 | -0.052 |
| $PO_4^{3-}$ | 880 nm | NA | -0.015 | -0.012 | -0.015 |

## 2.3.2 Daily IEM Loading Capacity Assessment

A daily IEM loading capacity assessment was performed to assess the IEMs' loading capacity simulating field conditions in the laboratory environment for 60 days with 5-day intervals. A mixed stock solution was prepared to represent an average 5-day-concentration of the months with the highest concentration recorded, during the baseline period, 7 mg $NH_4^+$-N/L, 12 mg $NO_3^-$-N/L, and 1 mg $PO_4^{3-}$-P/L, respectively. One AMI and one CMI (2 x 4 cm) were inserted in pre-labeled sample vials with 50 mL mixed stock solution. The samples were shaken for 24 h at 180 pm. After 24 h, the day-equivalent set of samples IEMS were extracted with 0.5 M HCl, shaken for two h at 180 rpm, and both extract and residual were frozen until analysis. The rest of the IEMS were transferred in new vials, the freshly mixed stock solution was added and shaken for 24 h following the process described above. Residual samples were frozen until analysis. The assessment was performed in triplicates.

It was observed that the $NH_4^+$ ions (Figure 2-6A) are adsorbed linearly for the first 35 days of incubation in the soil; for the following ten days, the CMI adsorbance capacity stays similar, and in the final ten days, the CMI adsorbance capacity is increased again. For the first 40 days, the CMIs could successfully adsorb over 99.09% of the

$NH_4^+$ ions suspended in the stock solution; the following days, the CMI adrsorbance remained over 94%. Furthermore, in the first 40 days, the difference between the stock solution and the extracts' concentration of $NH_4^+$-N were differing from 6.5-14%, with the overall difference reaching 36%, suggesting that the CMI do not release the whole amount of ions that initially were adsorbed on them.

Similar patterns were observed for the $NO_3^-$ ions adsorbance capacity (Figure 2-6B). The linear adsorbance pattern, the plateau, and the slight increase the final ten days being present with the only difference that the adsorbance linearity was present for five days longer than the $NH_4^+$-N. Moreover, for the first 40 days, the AMIs were able to adsorb over 99.99% of the $NO_3^-$-N ions suspended in the stock solution and down to 96.1% for the whole period. Furthermore, in the first 45 days, the difference between the stock solution and the extracts' concentration of $NO_3^-$ were differing from 1.6-10%, with the overall difference reaching 21%, suggesting that the AMIs are not releasing the whole amount of ions that initially were adsorbed on them.

Finally, for $PO_4^{3-}$-P ions, the adsorbance capacity (Figure 2-6C) was linear until the first 40 days, and after that, a dramatic decrease in the adsorbance capacity was observed. In that time frame, the AMIs successfully adsorbed from 94-99.9% of the $PO_4^{3-}$ ions suspended in the mixed stock solution, as indicated by the residuals concentrations, but for the 45-60 days, the adsorbance capacity was reduced to 55-82%. Moreover, in the first 45 days, the deviation between the stock solution and the extracts' concentration of $PO_4^{3-}$-P differed from 19-71%, with the extracts having higher concentrations than the initial stock solution. The deviation range was wider for 45-60 days, 24-81%, suggesting that the AMIs are getting saturated with $PO_4^{3-}$-P ions and cannot adsorb more.

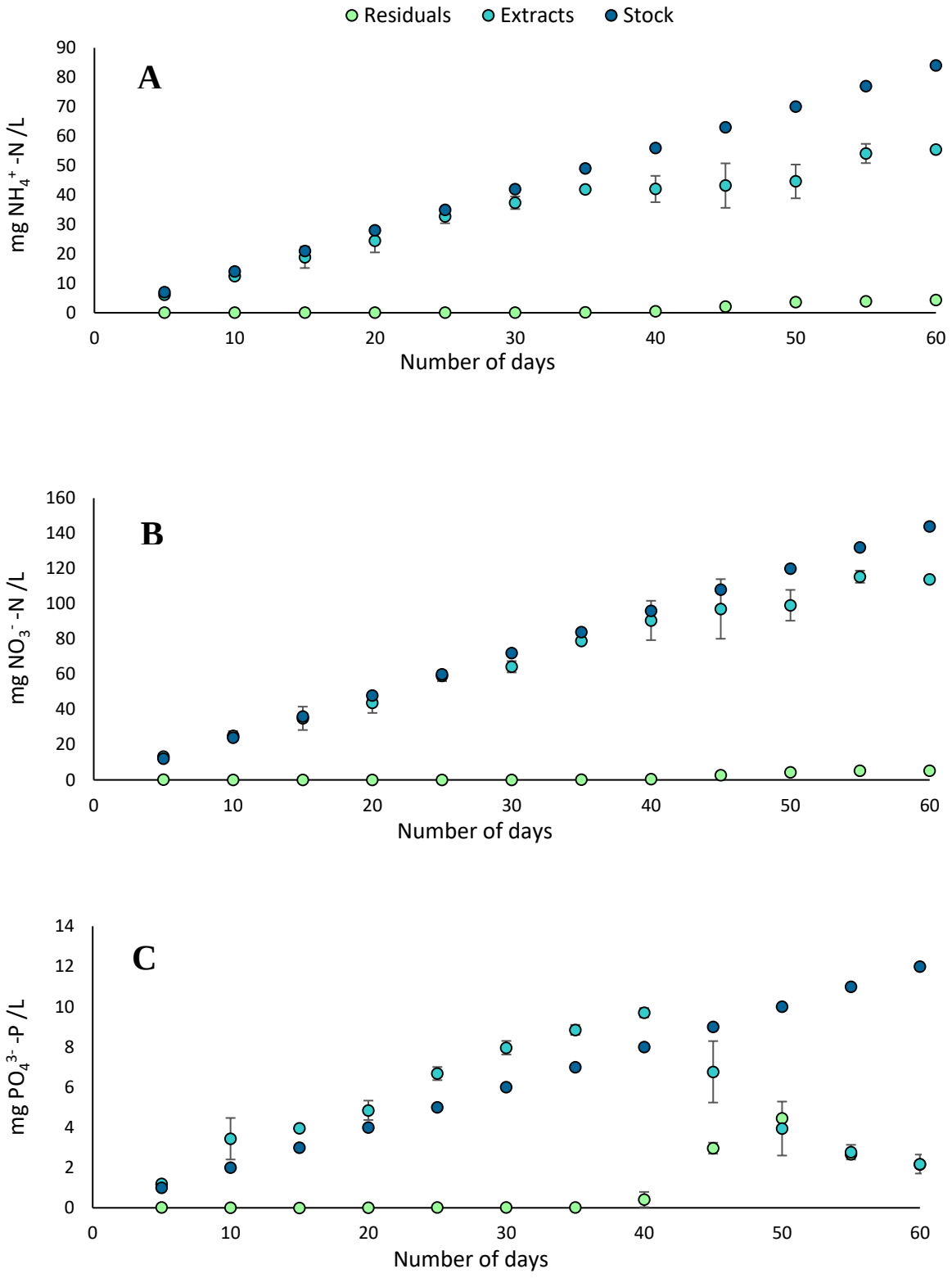


***Figure 2- 6:*** *Daily IEM loading capacity assessment for $NH_4^+$ (A), $NO_3^-$ (B), and $PO_4^{3-}$ (C). The light blue circles represent the extracted from IEMS concentrations, the dark blue circles represent the stock solution concentrations, and the green circles represent the residuals concentrations. The concentrations are in mg/L, and the error bars represent the standard deviation.*

### 2.3.3 Extraction time assessment

Another assessment was performed to assess the IEMs' extraction time of different concentrations. Three different concentration ranges were used (Low, Medium, High) for all three ions ($NH_4^+$-N: 7, 50, 80 mg/L; $NO_3^-$-N: 12, 80, 150 mg/L; $PO_4^{3-}$-P: 1, 7, 12 mg/L). One AMI and one CMI (2 x 4 cm) were inserted in pre-labelled sample vials with 50 mL mixed stock solution of each concentration range. The samples were shaken for 24 h at 180 rpm. After the 24 h, the IEMS were extracted with 0.5 M HCl, shaken for 2, 6, 10, and 24 h at 180 rpm, and both extract and residual were frozen until analysis. The assessment was performed in triplicates.

In Figure 2-7A, the different concentrations and extraction times are shown for $NH_4^+$. For the low concentration, the CMIs were able to adsorb 99.2-99.4% of the initial concentration, and the 2 h extraction was the most suitable one, providing 95% retrieval from the CMI. For the medium concentration, the CMI adsorbance capacity was similar to the one observed at the low concentration (99.2-99.4%), although the most suitable extraction time was 24 h, providing 93% retrieval. On the contrary, the CMI adsorbance capacity for the high concentration was significantly lower (73-79%), and the most suitable extraction time was 10 h, providing 81% retrieval.

In Figure 2-7B, the different concentrations and extraction times are shown for $NO_3^-$. For the low concentration, the AMIs were able to adsorb 100% of the initial concentration, and the 24 h extraction was the most suitable one, offering 92.5% retrieval from the AMIs. For the medium concentration, the AMI adsorbance capacity was similar to the one observed at the low concentration (99.8%), although the most suitable extraction time was the 10 h, offering 90% retrieval. Furthermore, the AMI adsorbance capacity for the high concentration was slightly lower (86-87%), and the most suitable extraction time was 24 h, providing 90% retrieval.

In Figure 2-7C, the different concentrations and extraction times are shown for $PO_4^{3-}$. The AMIs were able to adsorb 98-99% of the initial concentration for the low concentration, and the two h extraction was the most suitable one, offering 92% retrieval from the AMIs. For the medium concentration, the AMI adsorbance capacity was similar to the one observed at the low concentration (97-98%), although the most suitable extraction time was the 24 h, offering over 90% retrieval. However, for the high $PO_4^{3-}$-P concentration, all four extraction times performed relatively the same, and AMIs could not release the $PO_4^{3-}$P ions in the solution.

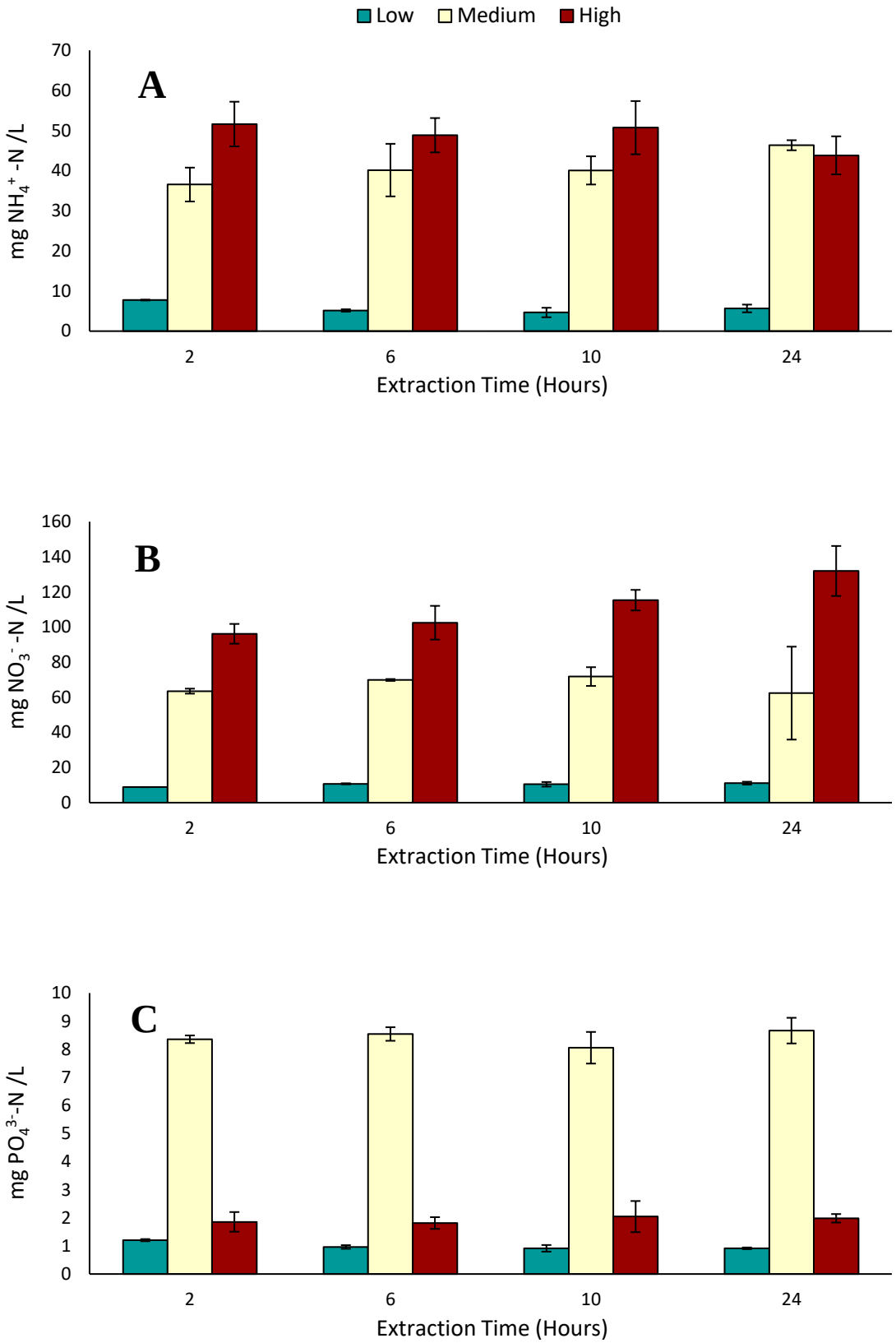


***Figure 2- 7:*** *Extraction time assessment for $NH_4^+$ (A), $NO_3^-$ (B) and $PO_4^{3-}$ (C). The green bars represent the lower concentrations, the yellow bars represent the medium concentrations, and the red bards represent the high concentrations for all three bioelements. The error bars represent the standard deviation.*

### 2.3.4 Soil analysis

After sampling, all samples were returned to the laboratory for further analysis. Before analysis, each core was weighted and separated into three horizons (O, A, and B). Each horizon was sieved (2 mm sieve), and roots and pebbles were removed. From each horizon, subsamples were taken for soil moisture, microbial biomass total organic C and N, $NH_4^+$, $NO_3^-$ and $PO_4^{3-}$, DNA extraction, and archiving purposes for future analysis. Figure 2-8 is the schematic representation of the initial steps taken in the laboratory for all soil campaigns. Detailed descriptions for all analyses can be found later in this Chapter. Root and DNA analyses were performed by Clare Ziegler and Aileen Baird, respectively, for their Ph.D. project purposes; thus, these datasets are not presented in this thesis.

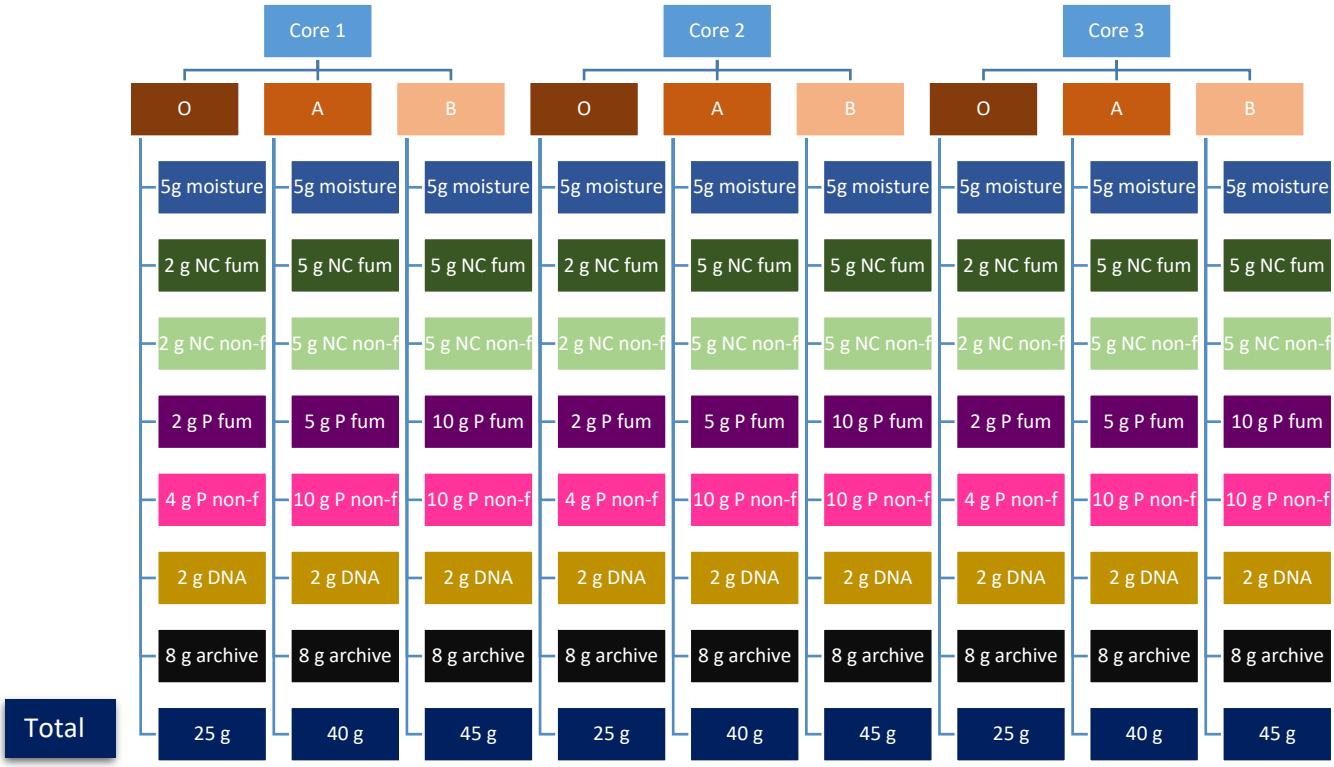


***Figure 2- 8:*** *Tree diagram depicting an example of the cores extracted in one array and the subsequent subsampling for the different analyses.*

### 2.3.4.1 Soil moisture and bulk density

Soil moisture was determined gravimetrically by drying a 5g subsample from each horizon, at 106 °C for 48 hours and was calculated as follows:

$$Soil\ moisture = \frac{(W_w + t) - (W_d + t)}{(W_d + t) - t} \qquad Equation\ 2-7$$

where $W_w$ is the weight of wet soil (g), $W_d$ is the weight of dry soil (g), and t is the tare's weight (g).

The soil moisture enabled the calculation of soil bulk density from:

$$\rho_d = \frac{M_s}{V_t} \qquad Equation\ 2-8$$

where $\rho_d$ is soil bulk density (g $cm^{-3}$), $M_s$ is the mass of dry soil (g) per core volume, and $V_t$ is the total volume of the soil core ($cm^3$).

### 2.3.4.2 Microbial biomass

To investigate the immobilization and mobilization of bioelements, specifically C and N, in the soil and their potential changes under elevated $CO_2$, four subsamples were taken from each horizon. Two of the subsamples mentioned above were fumigated with chloroform ($CHCl_3$) following the methods described at Brookes, Powlson, and Jenkinson, 1982; Brookes *et al.*, 1985; Beck *et al.*, 1997 and the other two were used

as a control for the $CHCl_3$ fumigation process. One of the chloroform fumigated-nonfumigated pair of subsamples was analysed for total organic C and N (*CN pair* hereafter), and the other pair (*$PO_4^{3-}$ pair* hereafter) was analysed for $PO_4^{3-}$-P (Table 2-3). Depending on the horizon and the subsequent analysis (CN or $PO_4^{3-}$), the subsamples' weights differed and can be found in the table. The $CHCl_3$ fumigated samples were put in a vacuum desiccator with a beaker containing 40 ml ethanol-free $CHCl_3$ and a moist paper towel to prevent the soils' desiccation. The desiccator was evacuated until the $CHCl_3$ boiled vigorously, and this process was repeated three times. On the fourth evacuation, once the $CHCl_3$ had boiled for two minutes, the valve was closed, and the desiccator was placed in the dark for 48 hours, preventing the $CHCl_3$ from breaking down. The non-$CHCl_3$ fumigated samples were also incubated in the dark next to the desiccator.

***Table 2- 3:*** *Summary of the different horizons' masses used for the $CHCl_3$ fumigation analysis.*

| | | Subsamples weights (g) | | |
|---|---|---|---|---|
| Analysis | | Horizons | | |
| **CN** | | O | A | B |
| | $CHCl_3$ fumigated | 2 | 5 | 5 |
| | $CHCl_3$ nonfumigated | 2 | 5 | 5 |
| **$PO_4^{3-}$** | $CHCl_3$ fumigated | 2 | 5 | 10 |
| | $CHCl_3$ nonfumigated | 4 | 10 | 10 |

### 2.3.4.3 Total organic C and N

Immediately after 48 hours of $CHCl_3$ fumigation, both $CHCl_3$ fumigated, and non-fumigated samples for the CN analysis were extracted with 10 ml of 0.5 M $K_2SO_4$,

shaken for 2 hours at 200 rpm, and centrifuged for 5 minutes at 3999 rpm. Subsequently, all samples were syringe-filtered with a 0.45 mm syringe filter. The samples were stored overnight in the fridge until their next day's analysis.

#### 2.3.4.4 Inorganic N

The non-$CHCl_3$ fumigated samples mentioned above were also analysed for soil extracted $NH_4^+$ and $NO_3^-$ by a continuous flow analyser (San ++ Continuous Flow Analyser, Skalar, Breda, The Netherlands) as described in section 2.3.1, with the only modification that 0.5 M $K_2SO_4$ was used for the standard preparation instead of deionised water and as a carrier to keep the matrix the same.

#### 2.3.4.5 Microbial P

Immediately after 48 hours of $CHCl_3$ fumigation, in both $CHCl_3$ fumigated and non-fumigated samples for the $PO_4^{3-}$ analysis, an AMI (2 x 4 cm size) and 15 ml of deionised water were inserted in each sample, as adapted from Sharpley (2000). The samples were shaken for 2 hours at 200 rpm. Subsequently, the AMIs were removed, washed, and extracted following the same process described in section *2.3.1 Soil bioavailable nutrients* with the modification that the HCl extraction lasted 24 hours instead of 2. The samples were analysed spectroscopically (UV-Vis Jenway 6850, Cole-Palmer, UK) as described in section 2.3.1.

## 2.4 Statistical Methods

All analyses were carried out using R 3.6.2 and Rstudio 1.2.5033 (RStudio Team, 2019). The mixed-effects models were performed using R package lme4 (Bates *et al.*, 2015), and the coefficient of determination was calculated using the R package MuMIn

(Barton, 2014). For all analyses, effects were considered to be strongly significant if the model-reported P-value < 0.05.

Many semi-empirical approaches are modeling soil respiration response to environmental variables due to the C flux's global significance between ecosystems and the atmosphere (Raich, Potter and Bhagawati, 2002). Soils are associated with high heterogeneity and do not abide under a single functional unit, as in the case of photosynthesis, in which the functional unit is the leaf (Kutsch, Bahn and Heinemeyer, 2010). In soils, various organisms and enzymes are decomposing a variety of chemical substances, and only a few soil microbes have been studied. Hence, soil respiration modelling often follows a holistic approach, modelling soil respiration responses as a whole.

The most commonly used semi-empirical modelling approaches are the exponential $Q_{10}$ relationship (Hoff, 1898), the Arrhenius equation, and its subsequent modification by Lloyd and Taylor (1994) or gap-filling strategies (Gomez-Casanovas *et al.*, 2013). However, both approaches include only soil temperature as a predictor variable of soil respiration; the $Q_{10}$ approach does not always provide the best fit of the soil respiration responses, while the Arrhenius might not apply under changing substrate availability circumstances (Kutsch, Bahn and Heinemeyer, 2010).

When studying soil respiration responses *in situ*, both biotic and abiotic factors, such as photosynthesis, soil moisture, and soil temperature, co-vary. Hence, when soil respiration variation is attributed to a single variable, background correlations with other variables can rumple the relationship. Moreover, our study includes unavoidably the spatial dimension since forest soils are heterogeneous. Thus, we concluded that the optimum approach that could describe the soil respiration responses was to use a

semi-empirical relationship in which soil moisture, soil temperature, soil respiration, and their interactions with $eCO_2$ were used to predict soil respiration responses. Moreover, our approach includes both the spatial and temporal elements in the soil respiration predictions.

## 2.4.1 Chapter 3 – Additional methodology

### 2.4.1.1 Environmental drivers and $R_s$

To assess the assess potential $eCO_2$ effect on environmental drivers, daily averages of the VWC and $T_s$ were analysed in a mixed-effect model framework treating $eCO_2$ as a fixed effect with 'array'. Data collected before the $eCO_2$ enrichment (up to 3rd April 2017) were analysed in a separate mixed-effects model framework to assess potential differences before $eCO_2$ enrichment due to soil's inherent spatial variability.

Before performing the mixed-effect models for investigating the effect of Rs' environmental variable$_s$, we needed to establish the variables' relationships with $R_s$. Linear and quadratic relationships between $R_s$ and VWC were tested, and linear and exponential relationships between $R_s$ and $T_s$. The best models were chosen based on the Akaike information criteria (AIC).

Mixed-effects models were also used to investigate the effect of environmental drivers, season, $eCO_2$, and their interactions on $R_s$, aggregated in daily averages. For this mixed-effects framework, the variables above ($eCO_2$, VWC, $T_s$, and season) were treated as fixed effects while 'array', 'collar/sensor', and 'number of days since the beginning of the measurements' were treated as random effects. Before the eCO2 enrichment (up to 3rd April 2017), data collected were analysed separately to assess

potential differences before $eCO_2$ enrichment due, presumably to spatial inhomogeneities in $R_s$.

The AIC tool was used to identify the two best-fitted models, and an ANOVA was used to determine which of the two models was the best fitted. Finally, the coefficient of determination ($R^2$) was calculated to assess the model's predictive capacity.

#### 2.4.1.2 Annual Rs estimates

Due to the experimental setup (measuring one ambient array and one treatment array for two weeks) and given the seasonal and temporal variability of $R_s$, a modelled approach was used to calculate the annual $R_s$. Daily averages of the $R_s$ data were estimated using the model of best fit from the *Environmental drivers'* section, predicting the $R_s$ of the rest of the arrays at all periods for the whole study period. VWC and $T_s$ from each array aggregated in daily averages were used as predictors. Subsequently, daily averages of observed and predicted $R_s$ values were merged, producing a complete $R_s$ dataset.

#### 2.4.1.3 Bioavailable nutrients

For nutrient analysis, the replication unit was the FACE array (n=3 for each of ambient and treatment), and all data were aggregated in monthly timestamps. The effect of $eCO_2$ on bioavailable nutrient concentrations was assessed using linear-mixed-effects models, for which 'array' and 'number of days since the beginning of the measurements' were random factors. Data collected before the $eCO_2$ enrichment were analysed separately to assess initial pretreatment differences. Subsequently, linear-mixed-effects models were undertaken with $CO_2$ and two covariates – $T_s$ and VWC –

and their interactions ($eCO_2$*$T_s$, $eCO_2$*VWC, and $eCO_2$*$T_s$ *VWC) as fixed effects to evaluate the role of soil conditions in responses to elevated $CO_2$. In this mixed-effects framework, ‘array’ and ‘number of days since the beginning of the measurements’ were used as random factors.

## 2.4.2 Chapter 4 – Additional methodology

### 2.4.2.1 Environmental drivers and $R_s$

To assess the assess potential $eCO_2$ effect on environmental drivers, daily averages of the VWC and $T_s$ were analysed in a mixed-effect model framework treating $eCO_2$ as a fixed effect with ‘array’. Before performing the mixed-effect models for investigating the effect of Rs' environmental variable$_s$, we needed to establish the variables’ relationships with $R_s$. Linear and quadratic relationships between $R_s$ and VWC were tested, and linear and exponential relationships between $R_s$ and $T_s$. The best models were chosen based on the Akaike information criteria (AIC).

Mixed-effects models were also used to investigate the effect of environmental drivers, season, $eCO_2$, and their interactions on $R_s$, aggregated in daily averages. For this mixed-effects framework, the variables above ($eCO_2$, VWC, $T_s$) were treated as fixed effects while ‘array’, ‘collar/sensor’, and ‘number of days since the beginning of the measurements’ were treated as random effects. Finally, the AIC tool was used to identify the two best-fitted models, and an ANOVA was used to determine which of these two models was finally the best. Moreover, the coefficient of determination ($R^2$) was calculated to assess the model's predictive capacity.

#### 2.4.2.2 Annual $R_s$ estimates

Due to the experimental setup (measuring one ambient array and one treatment array for two weeks) and Rs' seasonal and temporal variability, a modelled approach was used to calculate the annual $R_s$. Daily averages of the $R_s$ data were estimated using the best fit model from the *Environmental drivers'* section, predicting the $R_s$ of the rest of the arrays at all periods for the whole study period. VWC and $T_s$ from each array aggregated in daily averages were used as predictors. Subsequently, daily averages of observed and predicted $R_s$ values were merged, producing a complete $R_s$ dataset.

#### 2.4.2.3 Soil bioavailable nutrients

For nutrient analysis, the replication unit was the FACE array (n=3 for each of ambient and $eCO_2$), and all data were aggregated in monthly timestamps. The effect of $eCO_2$ on bioavailable nutrient concentrations was assessed using linear-mixed-effects models, for which 'array' and 'number of days since the beginning of the measurements' were random factors. Data collected before the $eCO_2$ enrichment (Pre-treatment) and Year 1 were analysed separately in Chapter 3.

Mixed-effects models were used to determine whether the relationships between $T_s$ and VWC with the nutrient availabilities were linear or exponential. The appropriate models were chosen via an ANOVA. Subsequently, mixed-effects models were undertaken with $eCO_2$ and three covariates – $T_s$, VWC, and P – and their interactions ($eCO_2$*$T_s$, $eCO_2$*VWC, and $eCO_2$*P) as fixed effects to evaluate the role of climatic conditions in responses to $eCO_2$. Utilising the Akaike Information Criterion (AIC) tool, the two models with the lowest AIC were chosen. An ANOVA was used to determine which model of the two mentioned above was finally the best-fitted one.

#### 2.4.2.4 Microbial biomass and inorganic N

Similarly, for the soil intensives' analysis, the replication unit was the FACE array (n=3 for each of ambient and $eCO_2$). We utilised again the linear-mixed-effects models to assess the $eCO_2$ effect on microbial biomass and inorganic N, for which 'array' and 'campaign' were random factors, while 'soil horizon', 'season' and '$eCO_2$' were fixed factors.

### 2.4.3 Chapter 5 – Additional methodology

Due to experimental design failure (fine root ingrowth in root exclusion collars), only descriptive statistics (e.g. average, minimum and maximum values) were performed for Chapter 5 for all the partition components of soil respiration ($R_h$, $R_a$, $R_r$, and $R_m$). The total soil respiration ($R_s$) was not investigated in Chapter 5 as it was extensively studied in Chapters 3 and 4. For more details, please see Chapter 5.

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# Chapter 3: Short-term carbon cycling and nutrient availability responses of a mature oak woodland under elevated $CO_2$

## 3.1 Abstract

Significant uncertainties are intertwined with future carbon (C) uptake and storage by terrestrial ecosystems. The University of Birmingham's Institute of Forest Research (BIFoR) was established in 2016, and one of its main scopes is to study the effects of elevated atmospheric $CO_2$ ($eCO_2$) on a temperate mature woodland utilizing its Free-Air $CO_2$ Enrichment (FACE) facility. BIFoR FACE is the first whole-ecosystem FACE experiment studying a mature deciduous temperate woodland and simulates global atmospheric concentrations expected to pertain ca. 2050. During the first year of $eCO_2$ enrichment, $eCO_2$ was observed to affect soil respiration positively, but the effect was not statistically significant. The annual estimates of soil respiration were stimulated by approximately 21% (1,341 vs. 1,624 g C $m^{-2}$ $y^{-1}$ in ambient and $eCO_2$ arrays, respectively). However, the $eCO_2$ effect was significant only when considering the synergic interaction of soil temperature and soil moisture.

Moreover, $NO_3^-$-N availability showed a prompt and sustained decrease, up to 37% in monthly averages, in $eCO_2$ arrays, whereas $eCO_2$ did not affect $NH_4^+$-N and $PO_4^{3-}$-P availability in the soil. The belowground responses observed in this study were more likely driven by increased carbon allocation belowground and utilisation from the extensive network of roots, ectomycorrhizal fungi, and soil heterotrophs. However, the ecological significance of these responses for mature ecosystems under $eCO_2$ and their persistence through time require further research.

## 3.2 Introduction

Anthropogenic $CO_2$ emissions have resulted in an over 50% increase in atmospheric $CO_2$ concentrations since the pre-industrial era (annual mean for 2019: 411.5 ppm, *Daily CO2*, 2019), and this rise is predicted to continue (Stocker *et al.*, 2013). According to future projections, the extent and the rate of this increase are not only dependent on the anthropogenic emissions (UNFCCC, 2005) but also on the capacity of ecosystems to take up carbon (C) and store it (Friedlingstein *et al.*, 2006; Friedlingstein *et al.*, 2014). The terrestrial biosphere plays a pivotal role in climate change and C cycling and is associated with the highest uncertainty regarding its response to (i.e., feedback on) environmental change (Lovenduski and Bonan, 2017; Quéré *et al.*, 2018).

Forest ecosystems are of high importance for the C cycle since it is estimated that ~ 860 ± 60 Pg C are currently being stored in the world's forests (Pan *et al.*, 2011). Northern temperate forests are the primary regulators (Schimel, Stephens and Fisher, 2015) of the terrestrial C sink, sequestering ~ 0.7 Pg C $y^{-1}$; one-third of the total uptake (Pan *et al.*, 2011). From a theoretical perspective, mature forests are close to equilibrium regarding their growth/turnover and litter inputs/soil respiration rates resulting in a comparatively constant C storage (Sousa, 1984). However, disturbances can cause this equilibrium to move, altering the C storage capacity of the ecosystem. One such disturbance is increasing atmospheric $CO_2$, which may 'fertilise' forest ecosystems, leading to an uptake of anthropogenic $CO_2$ into the plant and soil biomass (Zhu *et al.*, 2016). Although currently, forest ecosystems serve as a $CO_2$ sink (Pan *et al.*, 2011; Quéré *et al.*, 2018), the future projections of forest ecosystems' C storage capacity are highly uncertain (Ciais, Sabine and Bala, 2013).

Soil respiration ($R_s$) is the production of $CO_2$ from the soil when plant roots, microbes, hyphae, and soil fauna respire and is the second-largest flux of C between terrestrial ecosystems and the atmosphere (Raich and Schlesinger, 1992; Zhao *et al.*, 2017). Globally, it is estimated that soil respiration accounts for the release of 75-100 Pg C $yr^{-1}$, an order of magnitude larger than anthropogenic fossil fuel combustion (Boden, Marland and Andres, 2012). Hence, small changes in soil $CO_2$ effluxes may provoke significant changes in the net C balance, potentially shifting the C sink into a C source, at least temporarily, with substantial implications for the global C cycle (Cox *et al.*, 2000). Mainly, deciduous forests release approximately 1.18 Pg C $y^{-1}$ via the process of soil respiration (Warner *et al.*, 2019).

$R_s$ is one of the least-studied elements of the terrestrial C cycle due to spatial and temporal sampling limitations and the high cost of measurement instruments (Savage, Davidson, and Richardson, 2008). Moreover, $R_s$ is highly variable both temporally and spatially (Bond-Lamberty and Thomson, 2010), adding great uncertainty to global C cycle models. Although it is generally accepted that $R_s$ may be enhanced by elevated atmospheric $CO_2$, it is possible that the enhancement mentioned above may be negated by other constraints, making its role in climate feedback debatable (Qi, Xu and Wu, 2002; Davidson and Janssens, 2006). Understanding the nutrient mechanisms in mature forest ecosystems' capacity to sequester additional C is of great global concern and requires further investigation.

Many studies have shown that the magnitude and persistence of $CO_2$ fertilization effects on enhanced plant growth are linked with soil nutrient availability. The highest growth responses were seen in experiments where soil N availability was high (Reich, Hungate and Luo, 2006; Reich and Hobbie, 2013). Soil N availability is widely recognised as a crucial factor of productivity responses under elevated $CO_2$ (de Graaff

*et al.*, 2006; Reich, Hungate and Luo, 2006; Norby and Zak, 2011), with progressive N limitation predicted to be a critical mechanism limiting C fertilization responses over time (Luo *et al.*, 2004). Effects of elevated $CO_2$ on soil N availability have been reported to be both positive and negative (Zak *et al.*, 2000), and more specifically in FACE experiments, show no change (Finzi *et al.*, 2002; Zak *et al.*, 2003) or a decrease (Hovenden *et al.*, 2008; Lagomarsino *et al.*, 2008).

Contrary to the high interest that has been given to the effects of elevated $CO_2$ on the N cycle, impacts of elevated $CO_2$ on P availability, or both N and P availability, have been given less attention. Field studies that have investigated the effects of elevated $CO_2$ on P availability were conducted in combination with N fertilization of a subset of the FACE plots and have reported both increases (Khan *et al.*, 2010) and decreases (Lagomarsino *et al.*, 2008). The only FACE experiment investigating the impacts of elevated $CO_2$ on both N and P availability without nutrient fertilisation has reported increases in the availability of both nutrients under $eCO_2$ (Hasegawa, Macdonald and Power, 2016).

However, $eCO_2$ affects $R_s$ and nutrient availability via direct and indirect channels by affecting environmental variables, such as soil moisture (volumetric water content; VWC) and soil temperature ($T_s$). The $eCO_2$ effect on VWC is uncertain since studies have reported either small increases (Leuzinger and Korner, 2007; Manderscheid, Erbs and Weigel, 2014; Andresen *et al.*, 2018; Moser *et al.*, 2018), marginal increases, or no increase at all (Drake *et al.*, 2016). A potential rise in VWC will sequentially lead to a decrease in the topsoil availability of specific nutrients that are prone to leaching (McKinley *et al.*, 2009; Siemens *et al.*, 2012), an increase in plant C input to the soil, and faster decomposition due to alleviation of the water stress (Marhan *et al.*, 2010).

Moreover, increased VWC can alter microbial activity, C and N mineralization, and weathering (Kuzyakov *et al.*, 2019), which will impact $R_s$.

Although most of the previous FACE experiments were conducted in young stands or relatively homogenous plantations (King *et al.*, 2004), mature forests may respond differently to elevated $CO_2$. Old-growth forests cannot be assumed to respond in the same way as young stands. Old-growth forests may have thoroughly explored and exploited their belowground resources, limiting the possibility of increased C allocation belowground (Körner, 2006). Moreover, nutrient cycles in mature forests are more tightly coupled than in young forests (Zaehle *et al.*, 2014). Mature trees may also have less flexibility in nutrient use (Körner *et al.*, 2005).

We continuously measured Rs at hourly intervals across the first year of $CO_2$ enrichment in a mature deciduous temperate woodland in the present study. We collected approximately 40,000 high-frequency automated measurements, offering meticulous scrutiny of $R_s$ and its relationships with environmental drivers, including $eCO_2$. We also assessed the availability of ammonium ($NH_4^+$-N), nitrate ($NO_3^-$-N), and phosphate ($PO_4^{3-}$-P) at monthly intervals over the same period by deploying, collecting, and analysing over 1,000 ion-exchange membranes. Here we aim to establish the heterogeneity of $R_s$, VWC, $T_s$, and nutrient availability at BIFoR FACE before the $eCO_2$ enrichment and subsequently to answer the following questions:

- Does the $eCO_2$ affect $R_s$, soil moisture, and soil temperature on a mature oak woodland?
- How do bioavailable soil nutrients respond to $eCO_2$?

## 3.3 Methodology

### 3.3.1 Experimental site

The University of Birmingham's Institute of Forest Research (BIFoR) FACE is a temperate deciduous forest located within Mill Haft woodland in Staffordshire, England (52º 48' 3.6" N, 2º 18' 0" W). Mill Haft is part of Norbury Park Estate and has been woodland since pre-1881, appearing to be unchanged ever since. Mean precipitation during the overall studied period was 872.4 mm. Annual mean minimum and maximum temperatures for 2016 were 13, -3, and 32 ℃, respectively, and for 2017 were 10, -5.5, and 28 ℃.

A detailed site description can be found in (MacKenzie *et al.*, 2021). Briefly, the soil is brown earth cambisol with a sandy-clay texture and a mean soil pH of 4.5 at the top 10 cm. Mean soil total N and P in the top 10 cm are 0.28% and 18 mg P $kg^{-1}$, respectively, indicating that BIFoR is a low nutrient woodland, and total organic matter content is 3.67%.

The dominant overstorey species at the BIFoR FACE facility is old-growth (>160 years) *Quercus robur* (pedunculate oak), approximately 25 m in height. The woodland has a dense multi-layered canopy unaffected by constructing the FACE infrastructure (Hart *et al.*, 2019). The understorey is interspersed with *Corylus avellana* (common hazel), *Acer pseudoplatanus* (sycamore), *Ilex aquifolium* (holly), *Crataegus monogyna,* and *C. laevigata* (two native species of hawthorn).

### 3.3.2 BIFoR FACE facility

A detailed FACE facility description can be found in Hart *et al.*, (2019). Briefly, the BIFoR FACE facility comprises six 30 m diameter infrastructure arrays (elevated $CO_2$,

n = 3; ambient controls, n = 3) and three non-infrastructure arrays. Each $eCO_2$ array (+150 ppm above ambient) was paired with an ambient array at the early planning phase of the experiment after the baseline studies determined several forest characteristics (MacKenzie *et al.*, 2021). $eCO_2$ enrichment began on 3 April 2017 until 27 October 2017 (budburst to leaf fall, approximately) and will continue for each growing season until 2026. The fumigation system operated from 05:00 to 22:00, depending on the solar angle. During the $eCO_2$ enrichment period, $eCO_2$ arrays were within the 10% of target for 81.6% of the scheduled operation time and 20% of the target within 96.7% of scheduled operation time (Hart *et al.*, 2019). Contamination of the ambient arrays by $CO_2$ from the $eCO_2$ arrays was rare and short-lived, with control arrays being 10% of the control setpoint 98.8% of the time. Figure 3-1 shows the canopy greenness index from the PhenoCam installation date (May 2016) until Year 1 (December 2017).


***Figure 3- 1:*** *Canopy "greenness index" at BIFoR FACE measured from PhenoCam showing the 90th percentile of the daily green chromatic coordinate (GCC) of the canopy phenology nine months before the $eCO_2$ switch-on until the end of the first year of $eCO_2$ enrichment. Data are shown from May 2016 (9 months before the $eCO_2$ enrichment) until December 2017 (end of the first year of $eCO_2$ enrichment). The grey lines indicate gaps in the dataset due to technical failures of the PhenoCam, while the red lines enclose the period of $eCO_2$ enrichment (https://phenocam.sr.unh.edu/webcam/sites/millhaft/)*

## 3.3.3 Measurements

### *3.3.3.1 Soil respiration, Rs*

Three PVC (Drainage Superstore, CMO Ltd, Plymouth, UK) collars for monitoring the $R_s$ were established within each of the six infrastructure arrays (n = 3, $eCO_2$ arrays n = 3, ambient arrays). Each of these 20-cm-diameter collars was inserted 5 cm into the soil and permitted the Rs' measurement. The collars were installed in spring 2016 and left for establishment through the growing season; the soil collars were permanently installed to reduce soil disturbance by repeated measurements and ensure that the same soil was measured over time (King *et al.*, 2004).

Automated measurements of $R_s$ were taken simultaneously at 1-hour intervals at all three surface collars in a fumigated array and its paired ambient control array, using 20-cm-diameter long-term chambers interfaced with a multiplexer and an infra-red gas analyser (IRGA) (Li-8100-104 long-term chambers, Li-8150 multiplexer and Li-8100A IRGA; LI-COR). The observation length was 2 min with 20 s dead-band, a 15 s pre-purge, and a 45 s post purge, giving a total measurement cycle of 3 min 20 s. The measurements began on October 19, 2016 (5 months before the beginning of $eCO_2$ enrichment). We report data until December 31, 2017 (2 months after the switch-off of $eCO_2$ enrichment at the end of the first treatment growing season). The LI-COR systems were moved every two weeks between replicating paired eCO2 and ambient arrays and returning to the same array pair on a 4-week rotation.

All data went through quality checks before analysis using SoilFluxPro software, version 4.0.1 (LI-COR, Nebraska, USA). Data points with negative linear fluxes or data points with a linear flux coefficient of variation higher than 3.5 were removed from the dataset, as these were indicative of leaks in the chamber. Data points with a linear flux coefficient of variation between 1.5 and 3.5 were manually checked for assessing the

fit quality. Of 69,216 data points during the first year of operation, 39,557 data points were used for analysis. There was a moderate data loss (43%), primarily due to equipment failures (water condensation in the electric plates) and, to a lesser extent, due to poor-quality fits. Daily and monthly averages of these data are reported in this study in μmol $CO_2$ $m^{-2}$ $s^{-1}$, while annual averages are reported in g C $m^{-2}$ $y^{-1}$.

#### *3.3.3.2 Environmental drivers*

Soil volumetric water content (VWC) and soil temperature ($T_s$) were measured with permanently installed shallow CS655 probes inserted in the top 12 cm soil (Campbell Scientific, Logan, UT, USA). VWC probes were sited one metre from each of the three experimental $R_s$ collars in each array. The VWC data were recorded in 15-min intervals by a data logger in each plot (CR1000, Campbell Scientific) and are reported in daily averages.

The temperature sensitivity ($Q_{10}$) of $R_s$ was calculated using the following equations

$$lnR_s = kT + C$$

$$Q_{10} = e^{10k}$$

where k is the regression slope of ln-transformed respiration against measured temperature, and *C* is an empirical constant (Kutsch, Bahn and Heinemeyer, 2010). $Q_{10}$ was calculated for Pre-treatment and Year 1 separately to observe any $eCO_2$ effect.

#### *3.3.3.3 Biota available (bioavailable) inorganic N and P*

Ion-exchange resin membranes (Membranes International Inc., New Jersey, USA) were used to measure the available inorganic N, as ammonium ($NH_4^+$-N) and nitrate ($NO_3^-$-N), and P, as phosphate ($PO_4^{3-}$-P). Between May and October 2016, five anion exchange membranes (AMI 7001; 2 cm x 12 cm) and five cation exchange membranes (CMI 7000; 2 cm x 12 cm) were inserted in the top 12 cm of the soil within ~50 cm of each $R_s$ collar in each array. Following a statistical analysis of replicate variability, the sampling design was modified. From January 2017, three anion- and three cation-exchange resin membranes were inserted in the soil at eight locations within each array; three sets of each were located next to the $R_s$ collars, and the other five sets were randomly located within each array. Membranes were deployed in situ for approximately one month before retrieval. To assess the resin membranes' loading capacity, a capacity lab test was performed for 60 days, testing daily (see Chapter 2). Up to 40 days of resin membrane incubation, 99% of $NH_4^+$-N, $NO_3^-$-N, and $PO_4^{3-}$-P was absorbed in the membrane; beyond 40 days, the absorbance capacity decreased, but never to less than 90%.

Upon retrieval, membranes were washed thoroughly with deionised water to remove any soil particles and then extracted for $PO_4^{3-}$, $NH_4^+$ and $NO_3^-$ [modified from Rayment and Lyons, (2011) and Bowatte *et al.*, (2008), respectively]. Each membrane was extracted with 0.5 M HCl, shaken at 180 rpm for two hours, and then the membranes were removed from the solution and discarded. From January 2017 and onwards, the three membranes from each location were bulked together as one sample, and the same process was followed as described above.

Concentrations of $NH_4^+$ and $NO_3^-$ were analysed by a continuous flow analyser (San++ Continuous Flow Analyser, Skalar, Breda, The Netherlands), and concentrations of

$PO_4^{3-}$ were analysed by ultraviolet-visible spectroscopy (Jenway 6850) using the molybdenum blue method (Worsfold *et al.*, 2005). The data presented in this study are reported as monthly averages, per unit area of the membrane, in μg $NH_4^+$-N $cm^{-2}$ $d^{-1}$, μg $NO_3^-$-N $cm^{-2}$ $d^{-1}$, and μg $PO_4^{3-}$-P $cm^{-2}$ $d^{-1}$, respectively, and the ratios of these.

## 3.3.4 Statistical analysis

All analyses were carried out using R 3.6.2 and Rstudio 1.2.5033 (Rstudio Team, 2019). The mixed-effects models were performed using R package lme4 (Bates *et al.*, 2015), and the coefficient of determination was calculated using the R package MuMIn (Barton, 2014). For all analyses, effects were considered to be significant if the model-reported P-value < 0.05.

### *3.3.4.1 Environmental drivers and $R_s$*

Treatment effects were derived directly by comparing the daily means of $R_s$ for $eCO_2$ and ambient arrays using a one-way ANOVA. The Pre-treatment period and Year 1 were assessed separately to establish potential significant differences before $eCO_2$ enrichment and significant treatment effects, respectively. Interactions between $eCO_2$ and environmental drivers (VWC and $T_s$) were evaluated using a two-way ANOVA for both periods. The more complicated interactions between both environmental drivers were assessed using a three-way ANOVA. However, as described in *Chapter 2*, trying to investigate treatment effects in such dataset, where we have clustered repeated measurements across time, there is both spatial and temporal variability to account for, without compromising the real variability and dealing with potential imbalances in the number of measurements (very common for field datasets as such), mixed-effects

models are more advantageous than ANOVA. Hence in all mixed-effects models performed, 'array,' 'location within the array,' and 'number of days since the beginning of the experiment' to account for the correlations between repeated measurements within each collar/array were added as random effects. All mixed-effects models performed in this Chapter daily averages of $R_s$ (per collar), VWC, and $T_s$ (per sensor, respectively) were used.

Mixed-effects models were used to investigate potential pre-$eCO_2$ enrichment differences in the pre-assigned as 'ambient' and 'elevated' arrays. Although there was no $eCO_2$ enrichment during the Pre-treatment period, please allow been named as such for easier understanding. $eCO_2$, its interaction with VWC and $T_s$, and the complex interaction between all three variables were used as fixed effects. The relationships between $R_s$ and VWC as well as $R_s$ and $T_s$ were linear during the Pre-treatment period. Akaike Information Criteria (AIC) tool was used to determine which of the models mentioned above was most likely the best model for this dataset. The two models with the lowest AIC score were compared using the ANOVA[1] function to compute the Chi-squares between the two models for investigating the model fit. Finally, the coefficient of determination ($R^2$) was calculated to assess the variance captured by the model.

Similarly, the same principles as described above were used for investigating the $eCO_2$ effects during Year 1 of $eCO_2$ enrichment at BIFoR FACE. $eCO_2$, its interaction with VWC and $T_s$, and the complex interaction between all three variables were used as fixed effects. However, during Year 1, only Rs and Ts' relationship was linear, whereas $R_s$ and VWC was quadratic. The AIC tool was utilised again to choose the

[1] Although the function is called ANOVA it is a likelihood ration test (LRT) and not to be confused with the statistical method.

most likely best model amongst the ones mentioned above, while the ANOVA function was used for concluding the best-fitted model.

#### *3.3.4.2 Bioavailable nutrients*

For nutrient analysis, the replication unit was the FACE array (n=3 for each of ambient and $eCO_2$), and all data were aggregated in monthly timestamps. The effect of $eCO_2$ only on bioavailable nutrient concentrations was assessed using linear-mixed-effects models, for which 'array' and 'number of days since the beginning of the measurements' were random factors. Data collected before the $eCO_2$ enrichment were analysed separately to assess initial pretreatment differences. Subsequently, linear-mixed-effects models were undertaken with $CO_2$ and two covariates – $T_s$ and VWC – and their interactions ($eCO_2$*$T_s$, $eCO_2$*VWC, and $eCO_2$*$T_s$ *VWC) as fixed effects to evaluate the role of soil conditions in responses to elevated $CO_2$. In this mixed-effects framework, 'array' and 'number of days since the beginning of the measurements' were used as random factors.

### *3.3.5 Estimate of annual Rs*

Due to the experimental setup (measuring one ambient array and one $eCO_2$ array for two weeks) and given the seasonal and temporal variability of $R_s$, a modelled approach was used to calculate the seasonal $R_s$, utilising the best-fitted models described in section *3.3.4.1 Environmental drivers and $R_s$*. Daily averages of the $R_s$ data were estimated using the best-fitted models for Pre-treatment and Year 1, predicting the $R_s$ of the rest of the arrays at all periods for the whole study period. VWC and $T_s$ from each array aggregated in daily averages were used as predictors. Subsequently, daily

averages of observed and predicted $R_s$ values were merged, producing a complete $R_s$ dataset.

## 3.4 Results

### 3.4.1 $R_s$ response to environmental drivers and $eCO_2$

$R_s$ exhibited high variability throughout the study period, following seasonal patterns related to VWC, $T_s$, and precipitation patterns (Fig. 3-2). The pre-treatment period started in mid-autumn (19th October) 2016 and lasted until the early spring (2nd April) of 2017. During that period, no $eCO_2$ was introduced in the woodland; however, all arrays were pre-assigned as 'ambient' and '$eCO_2$'. The dashed lines on Fig. 3-2 enclose the $eCO_2$ enrichment period (3rd April – 27th October 2017; *Year 1* hereafter).

$R_s$ increased during the growing season (concurring with $eCO_2$ enrichment) in both ambient and $eCO_2$ treatments, as the $T_s$ increased and VWC decreased. Both daily and monthly averages indicated that Rs was frequently higher in the $eCO_2$ arrays. The greatest differences appeared to be during August and September 2017 (August 3.74 µmol $CO_2$ $m^{-2}$ $s^{-1}$ in ambient vs. 4.89 µmol $CO_2$ $m^{-2}$ $s^{-1}$ in $eCO_2$; September 2.98 µmol $CO_2$ $m^{-2}$ $s^{-1}$ in ambient vs. 4.34 µmol $CO_2$ $m^{-2}$ $s^{-1}$ in $eCO_2$). Precipitation was observed to lead to a pulse effect on $R_s$ commensurate with an increase in VWC. Both VWC and $T_s$ had a strong and clear seasonal pattern, while precipitation was sporadic and highly variable (Fig. 3-2b, c).

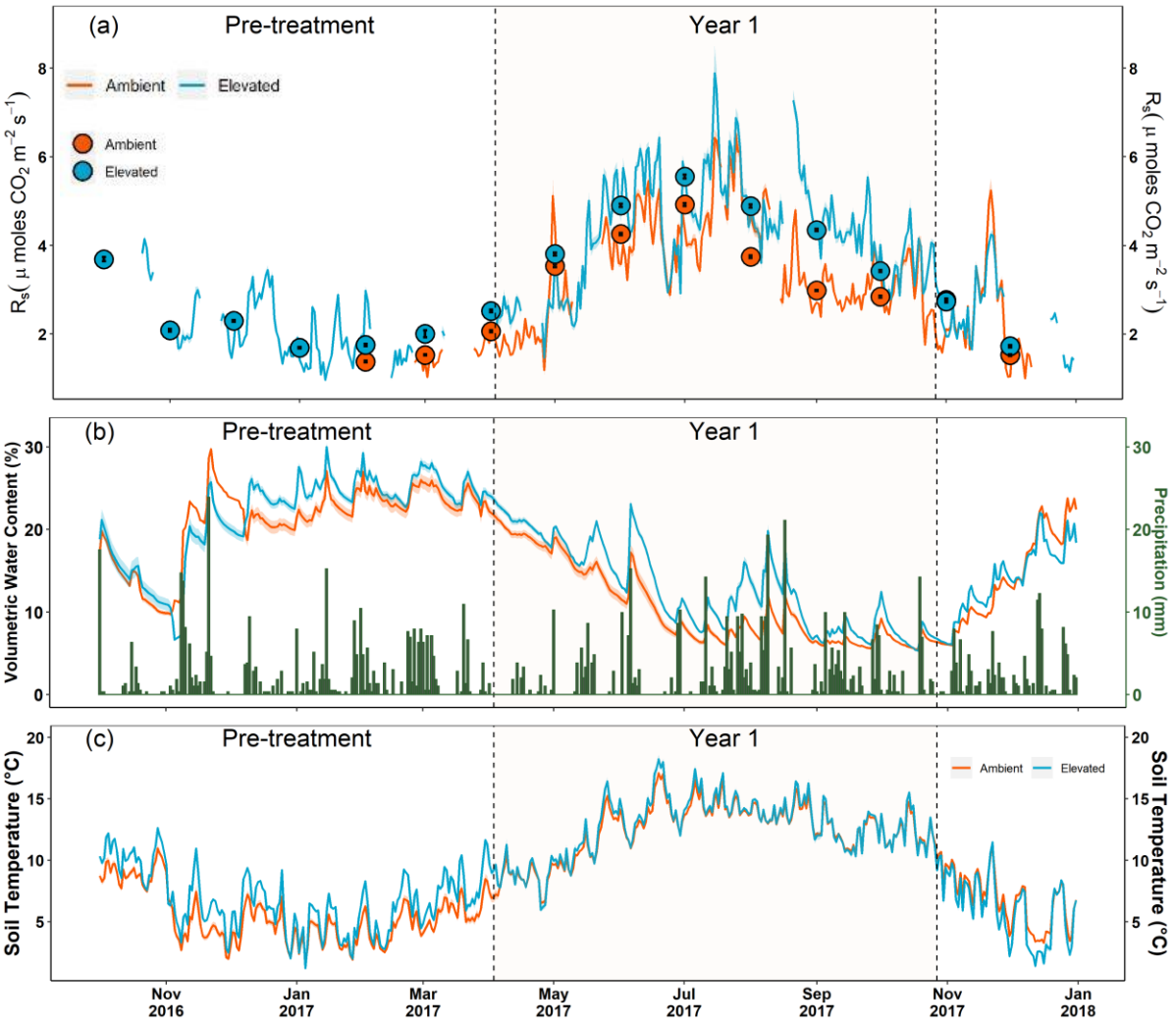


***Figure 3- 2:*** *Seasonal variation of $R_s$ (a), VWC and precipitation (b), and $T_s$ (c) at BIFoR FACE during the Pre-treatment and first year of $eCO_2$ enrichment (October 2016 – October 2017). The blue coloured lines/solid points denote the $eCO_2$ arrays, while the orange lines/solid points denote the ambient arrays. The lighter coloured ribbons around the lines and the error bars indicate the SE for $R_s$, VWC, and $T_s$. All data describe daily averages, except the solid points in (a), which reflect the $R_s$ monthly averages. The vertical dashed lines enclose the period when the $eCO_2$ enrichment was switched on (Year 1, 3rd April – 27th October 2017), whereas the period before reflects the Pre-treatment period (19th October 2016 – 2nd April 2017).*

We also investigated the $R_s$ response to rain events during Year 1 at BIFoR FACE (Fig. 3-3). For example, we present approximately nine rain events for 2.5 months (24th July – 1st August 2017). $R_s$ responded rapidly to rain events, and the magnitude of the $R_s$ rate increase was dependent on the conditions of the soils before the event. The drier and warmer the soils were before a rain event, the greater the increase on $R_s$.

Two rain events are studied here as an example; both events were chosen as they happened while the $R_s$ measurements were already in that location for a few days at least. Thus any potential $R_s$ difference observed can be attributed to the rain events rather than the $R_s$ spatial variability. For example, Event A, a small rain event (1 mm) in mid-July, almost doubled the $R_s$ under both ambient and $eCO_2$ conditions from ~ 3 to 6 and from ~ 4 to 8 μmol $CO_2$ $m^{-2}$ $s^{-1}$, respectively. Before this event, the soils at BIFoR FACE were warm and dry, having received less than 0.3 mm precipitation during the previous 11-day-period. However, no further increase on $R_s$ was observed during greater rain events (Event B end of July), when the soils were wetter because precipitation events were more frequent and the soil temperatures were lower.

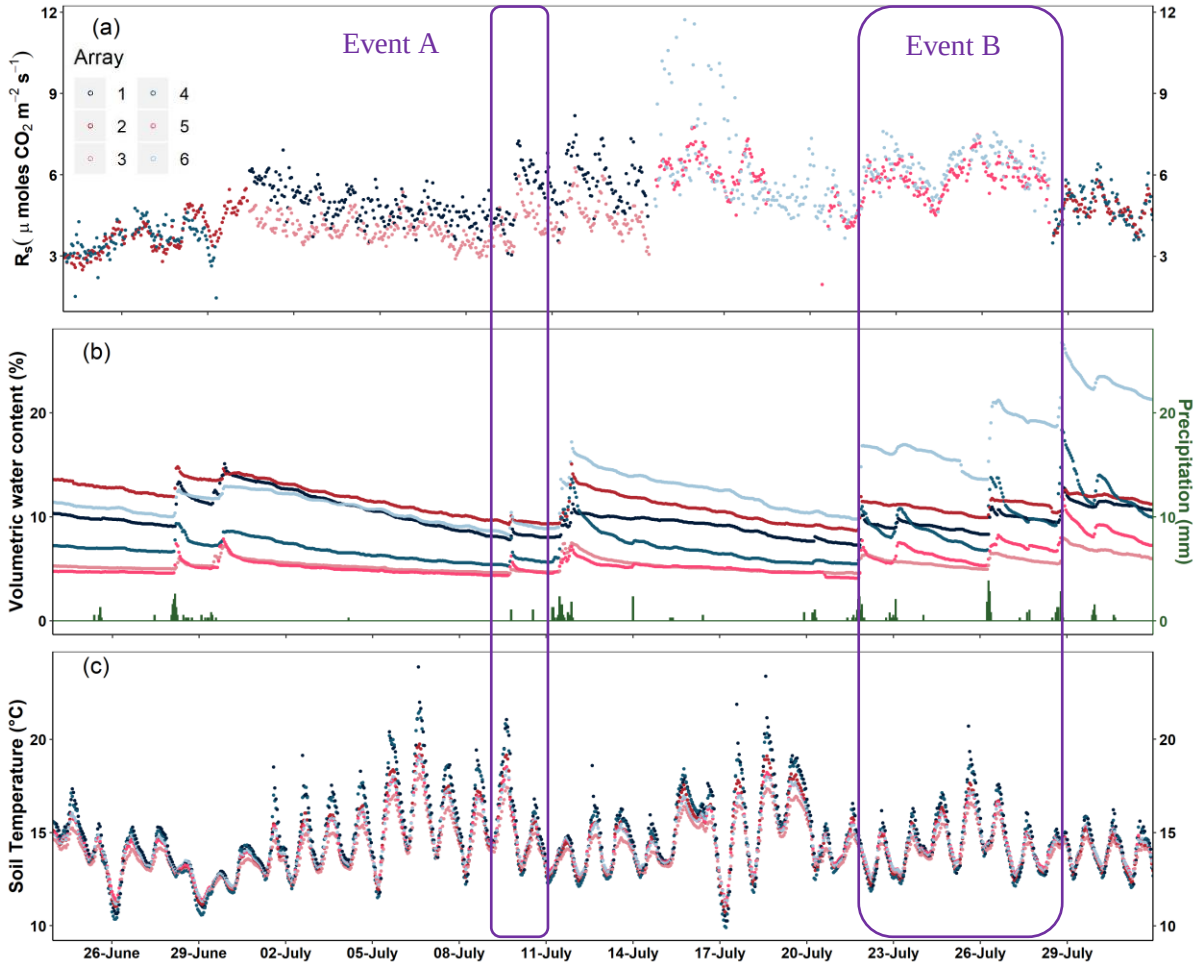


***Figure 3- 3:*** *(a) Example responses of $R_s$ to precipitation events during Year 1 of $eCO_2$ enrichment at BIFoR FACE. Warm colours reflect ambient arrays, while cold colours reflect $eCO_2$ arrays. Note that not all arrays were measured for $R_s$ simultaneously; instead, an ambient and an $eCO_2$ array were measured for a two-weekly period. (b) VWC (lines) and precipitation (bars), and (c) $T_s$ during the same period. All data presented in hourly averages.*

Table 3-1 shows the average (mean ± standard deviation), minimum and maximum VWC, $T_s$, and $R_s$ during Pre-treatment and Year 1 (2017), as well as the number of observations and the $Q_{10}$ values both ambient and $eCO_2$ arrays. The mean VWC, $T_s$, and $R_s$ were slightly higher in $eCO_2$ arrays during both Pre-treatment and Year 1, with the variables having a greater range and variability in $eCO_2$ arrays. $Q_{10}$ was higher in ambient arrays before and after the $eCO_2$ enrichment (Pre-treatment: 2.96 in ambient vs. 2.66 in $eCO_2$ arrays; Year 1: 3.08 in ambient vs. 2.64 in $eCO_2$).

Simple mixed-effects models were used to investigate significant differences in daily VWC, $T_s$, $R_s$ between ambient and $eCO_2$ arrays for both Pre-treatment and Year 1. All three variables (VWC, $T_s$, $R_s$) were statistically similar between the pre-assigned as 'ambient' and '$eCO_2$' arrays during the Pre-treatment period (p-value 0.78, 0.38, and 0.4, respectively; Table 3-1), as well as during Year 1 (p-value 0.35, 0.15 and 0.29, respectively; Table 3-1).

***Table 3- 1:*** *Mean, number of observations ('n'), minimum and maximum VWC, $T_s$, and $R_s$ for Pre-treatment and Year 1 in ambient and $eCO_2$ arrays based on daily averages (mean ± sd), as well as $Q_{10}$ values and p-values from simple mixed-effects models. Significance values are based on the mixed-effects model approach with eCO2 as the main effect and array, location within an array, and the number of days since the beginning of the measurement as random effects.*

| Variable | Mean | | Minimum | | Maximum | | n | | p-value |
|---|---|---|---|---|---|---|---|---|---|
| | Ambient | $eCO_2$ | Ambient | $eCO_2$ | Ambient | $eCO_2$ | Ambient | $eCO_2$ | |
| *Pre-treatment* | | | | | | | | | |
| VWC | 21.00±4.57 | 21.76±5.18 | 9.75 | 6.63 | 29.74 | 30.03 | 184 | 184 | 0.78 |
| $T_s$ | 5.47±2.13 | 6.80±2.55 | 1.75 | 1.23 | 10.97 | 12.62 | 184 | 184 | 0.38 |
| $R_s$ | 1.55±0.26 | 2.07±0.71 | 1.03 | 0.97 | 2.09 | 4.14 | 24 | 104 | 0.40 |
| $Q_{10}$ | 2.96 | 2.66 | | | | | | | |
| *Year 1* | | | | | | | | | |
| VWC | 10.60±4.80 | 12.93±5.20 | 5.48 | 5.35 | 21.75 | 23.68 | 208 | 208 | 0.35 |
| $T_s$ | 12.39±2.28 | 12.66±2.43 | 6.51 | 5.98 | 17.08 | 18.23 | 208 | 208 | 0.15 |
| $R_s$ | 3.51±1.15 | 4.32±1.23 | 1.19 | 1.47 | 6.63 | 7.89 | 190 | 195 | 0.29 |
| $Q_{10}$ | 3.08 | 2.64 | | | | | | | |

The synergic effects of $eCO_2$ treatment on $R_s$ with the environmental drivers (VWC and Ts) were also explored using more complex models that incorporated interaction effects. The dual impact of $eCO_2$ and each environmental driver and their triadic interaction can be found in Table 3-2. During Pre-treatment, no interaction was statistically significant; however, the model with the lowest AIC was the $eCO_2$*$T_s$. This model explained 64% of the observed variability on $R_s$. The model was validated for homoscedasticity, normality, and linearity of residuals, and linearity of the random effects (see section 3.8 Supplementary Material, Figure S3-2 and Table S3-5 for the model's coefficients).

During Year 1, the $eCO_2$*$T_s$ interaction and the eCO2*VWC*Ts interaction were statistically significant (p-value 0.008 and <0.001, respectively). These two interaction models were also the ones with the lowest AIC and could explain 61 and 63% of Rs' variability. The ANOVA function was used to determine which of the two models was the best fitted; the latter model was the best one (P-value < 0.001). Finally, the model was validated for homoscedasticity, normality, linearity of residuals, and linearity of the random effects (see section 3.8 Supplementary Material, Figure S3-3, and Table S3-5 the model's coefficients).

***Table 3- 2:*** *Analysis of Rs and its drivers' variance during Pre-treatment and Year 1 at BIFoR FACE. The degrees of freedom for each explanatory variable is 1, P < 0.1 is shown in bold.*

| Predictors | $DF_{den}$ | F-value | p-value | $R_c^2$ | AIC |
|---|---|---|---|---|---|
| *Pre-treatment* | | | | | |
| $eCO_2$ | 2.83 | 1.90 | 0.27 | 0.68 | 735.70 |
| $eCO_2$*VWC | 9.37 | 0.18 | 0.68 | 0.66 | 700.16 |
| $eCO_2$*$T_s$ | 296.34 | 1.88 | 0.17 | 0.64 | 616.58 |
| $eCO_2$*VWC*$T_s$ | 323.79 | 0.08 | 0.78 | 0.63 | 645.74 |
| *Year 1* | | | | | |
| $eCO_2$ | 4.02 | 1.50 | 0.29 | 0.31 | 4217.25 |
| $eCO_2$*VWC | 1170.3 | 1.21 | 0.27 | 0.50 | 4002.80 |
| $eCO_2$*$T_s$ | 1155.9 | 6.92 | **0.008** | 0.61 | 3540.43 |
| $eCO_2$*VWC*$T_s$ | 1162.06 | 35.02 | **<0.001** | 0.63 | 3530.30 |

*Where $DF_{den}$: denominator's degrees of freedom, F-value: F-test determination, $R_c^2$: coefficient of determination and AIC: Akaike Information tool*

Utilising the models' from above, we were able to estimate the $R_s$ by imputing data for the arrays that were not measured each fortnight or periods where no data existed and create a "complete" dataset. Thus, at all times, we have n=3 with two arrays being predicted values and the third one being the observed values for ambient and $eCO_2$ arrays, respectively.

Figure 3-4 shows the $R_s$ response to VWC and $T_s$ in both ambient and $eCO_2$ arrays using daily averages of the "gap-filled" dataset. $R_s$ in ambient arrays increased as $T_s$ increased, and VWC decreased, with the highest values observed at high $T_s$ and low VWC (Fig. 3-4a). In low temperatures, $R_s$ were similar regardless of VWC, indicating that $T_s$ was a limiting factor. In $eCO_2$ arrays, the highest $R_s$ were observed when both $T_s$ and VWC increased; however, the VWC range in $eCO_2$ arrays was much greater than in ambient arrays (Fig. 3-4b).

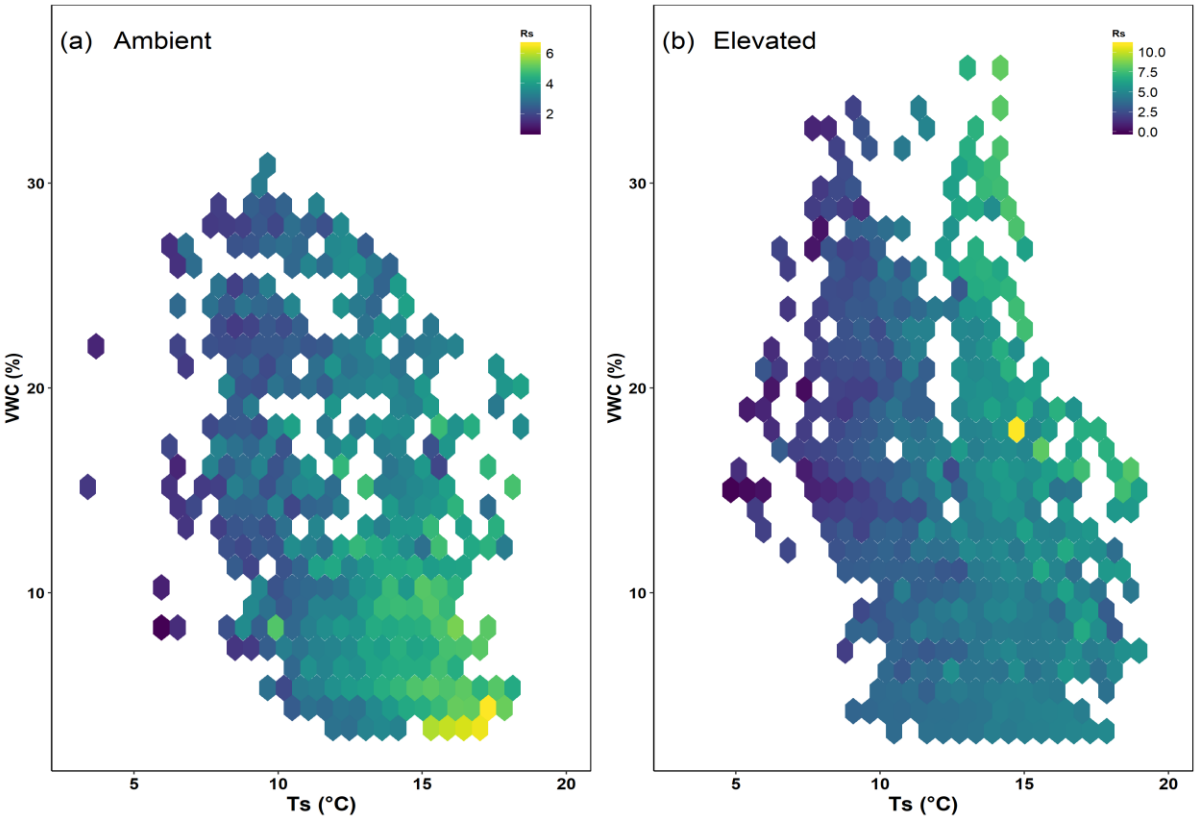


**Figure 3- 4:** *$R_s$ response to $T_s$ and VWC in ambient (a) and $eCO_2$ (b) conditions at BIFoR FACE during the first year of operation. Hexagons reflect daily averages per collar of the "gap-filled" dataset utilising the model's prediction.*

### 3.4.2 Estimates of annual Rs

As mentioned in the section above, the best-fitted models were utilised to predict Rs' missing values for the studied period. Figure 3-5a and b show the observed $R_s$ values against the predicted $R_s$ values in ambient and $eCO_2$ arrays during both Pre-treatment and Year 1 of $eCO_2$ enrichment at BIFoR FACE. Note that in Fig. 3-5a during the Pre-treatment period, the model cannot capture the temporal variability due to missing data. The observed values are daily averages of one array each fortnight, whereas the predicted values are daily averages of three arrays for ambient and $eCO_2$ arrays, respectively. The objective of the model was to "fill the gaps" between $R_s$

measurements with defensible estimates. Observed and predicted $R_s$ were strongly correlated (Fig. 3-5c; $p<0.001$ for both ambient and $eCO_2$ arrays; $R = 0.84$ and 0.88, respectively); the equation's slope was 1 and 0.96 for ambient and $eCO_2$ arrays, respectively.

Rs' mean annual flux was estimated as the predicted and observed daily averages of $R_s$ data above (Fig. 3-5d). The mean annual (i.e., October 2016- November 2017) $R_s$ at BIFoR FACE in ambient arrays was 1,341 ± 21 g C $m^{-2}$ $y^{-1}$ (mean±se), while in $eCO_2$ arrays was approximately 21.1% higher (1,624 ± 24 g C $m^{-2}$ $y^{-1}$; mean±se).

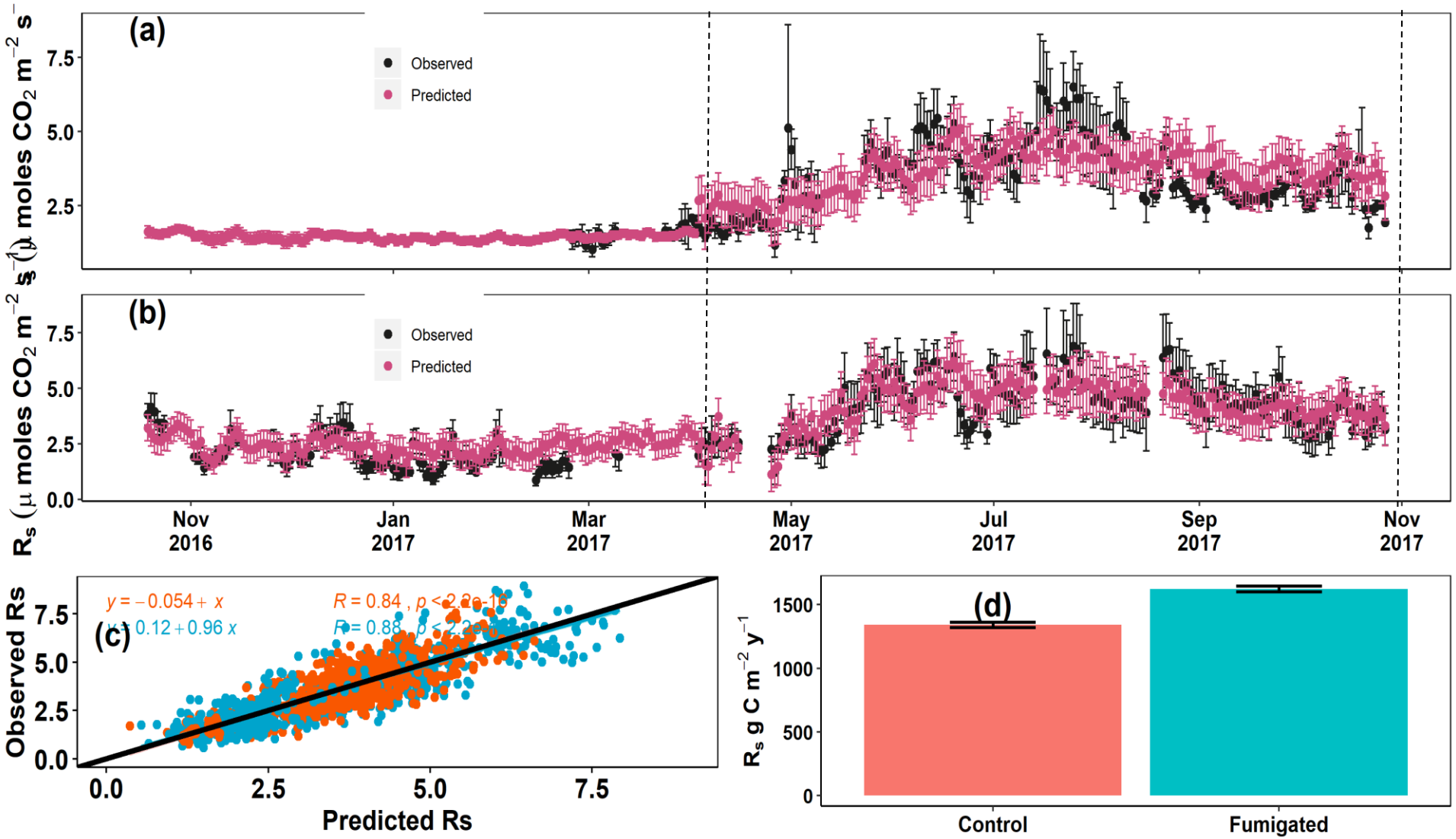


***Figure 3- 5:*** a) Observed and predicted $R_s$ measurements under ambient conditions, b) Observed and predicted $R_s$ measurements under $eCO_2$ conditions. The black solid points denote the daily-average (mean ± sd) observed $R_s$ measurements, while the pink solid points denote the predicted $R_s$ values. The dashed lines enclose the Year 1 of $eCO_2$ enrichment. c) Predicted and observed $R_s$ correlation for $eCO_2$ (blue line) and ambient (orange line) arrays. The blue solid points denote the $eCO_2$ arrays, while the orange solid points denote the ambient arrays. d) Mean annual $R_s$ in g C $m^{-2}$ $y^{-1}$ in ambient (orange) and $eCO_2$ (blue) arrays during the first year at BIFoR FACE. The error bars denote the standard error.

### 3.4.3 Biota available (bioavailable) inorganic N and P

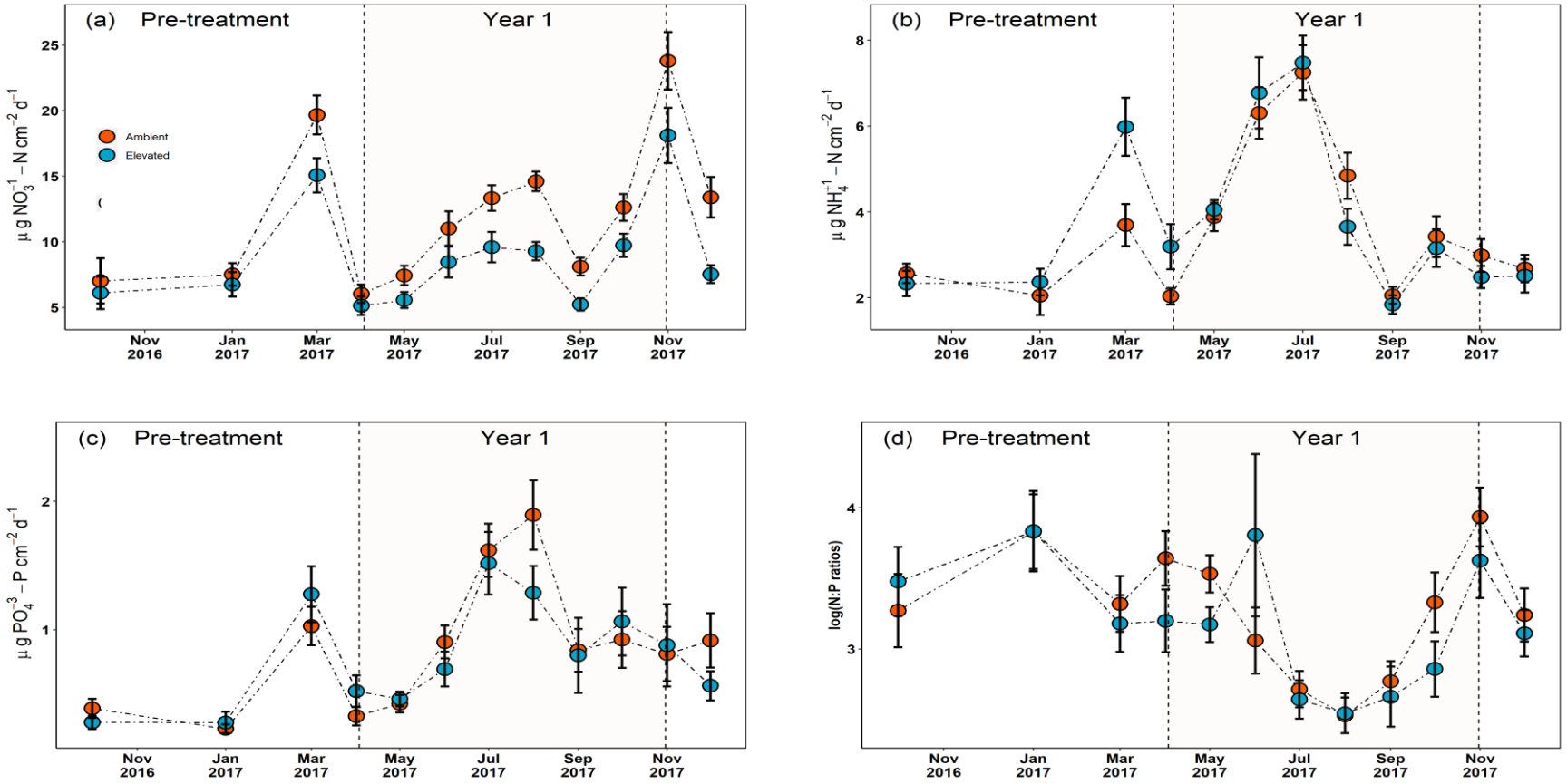


**Figure 3- 6:** *Temporal changes in ion exchange resin membranes (a) $NO_3^-$-N, (b) $NH_4^+$-N and (c) $PO_4^{3-}$-P concentrations, (d) log(N:P ratios), (mean ± se, n = 3). The orange solid points reflect the nutrient availability in the ambient arrays, whereas the blue solid points reflect the nutrient availability in the $eCO_2$ arrays. The shaded area denotes eCO2 treatment (Year 1, 3rd April – 27th October 2017), whereas the period before reflects the Pre-treatment period (1st October 2016 – 2nd April)*

All three soil bioavailable nutrients ($NO_3^-$-N, $NH_4^+$-N, and $PO_4^{3-}$-P) exhibited strong seasonality, with availability increasing as the $T_s$ increased and VWC decreased (Fig. 3-6a, b, and c). All three bioavailable nutrients had low availabilities at the beginning of the $eCO_2$ enrichment (April 2017; $NO_3^-$-N 6.0±0.7 in ambient vs. 5.1±0.7 μg $NO_3^-$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays; $NH_4^+$-N 3.9±0.3 in ambient vs. 3.2±0.5 μg $NH_4^+$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays; $PO_4^{3-}$-P 0.3±0.1 in ambient vs. 0.5±0.1 μg $PO_4^{3-}$-P $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays), while their highest availabilities were observed in summer for $NH_4^+$-N (July 2017; 7.3±0.6 in ambient vs. 7.5±0.6 μg $NH_4^+$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays) and $PO_4^{3-}$-P (August 2017; 1.9±0.3 in ambient vs. 1.3±0.2 μg $PO_4^{3-}$-P $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays) and in autumn for $NO_3^-$-N (November 2017; 23.8±2.2 in ambient vs. 18.1±2.1 μg $NO_3^-$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays).

$NO_3^-$-N had the lowest availability at the beginning of $eCO_2$ enrichment (April 2017) and increased as the growing season progressed (Fig. 3-6a). The $eCO_2$ arrays had persistently lower $NO_3^-$-N availability throughout the $eCO_2$ enrichment with the greatest difference observed in August 2017 (14.6±0.8 in ambient vs. 9.3±0.7 μg $NO_3^-$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays, respectively). $NH_4^+$-N and $PO_4^{3-}$-P exhibited a similar pattern response in both ambient and $eCO_2$ arrays, with no great differences observed in their availability between ambient and $eCO_2$ arrays. Accordingly, N:P ratios decreased as the availability of the nutrients increased.

***Table 3- 3:*** *Soil nutrients availability and N:P ratios during the Pre-treatment and Year 1 at ambient and $eCO_2$ arrays (mean ± sd), and summary of mixed-effects models of the $eCO_2$ effect on nutrient availability. All nutrients units are per unit area of membrane in µg $cm^{-2}$ $d^{-1}$(refer to section 3.3.4.2 Bioavailable nutrients for method). p-value < 0.1 is shown in bold.*

| | | | $eCO_2$ effect | | | |
|---|---|---|---|---|---|---|
| **Variable** | **Ambient** | **$eCO_2$** | **$DF_{num}$** | **$DF_{den}$** | **F-value** | **p-value** |
| *Pre-treatment* | | | | | | |
| $NO_3^-$ - N | 11.4 ± 7.0 | 9.1 ± 4.7 | 1 | 16 | 0.64 | 0.44 |
| $NH_4^+$ - N | 2.8 ± 1.1 | 3.5 ± 1.9 | 1 | 16 | 0.99 | 0.33 |
| $PO_4^{3-}$ - P | 0.6 ± 0.4 | 0.6 ± 0.7 | 1 | 16 | 0.05 | 0.82 |
| log(N:P) ratio | 3.5 ± 0.7 | 3.5 ± 0.9 | 1 | 4 | 0.02 | 0.89 |
| *Year 1* | | | | | | |
| $NO_3^-$ - N | 10.4 ± 3.6 | 7.3 ± 2.9 | 1 | 38 | 9.08 | **0.005** |
| $NH_4^+$ - N | 4.3 ± 2.1 | 4.9 ± 2.0 | 1 | 38 | 0.202 | 0.66 |
| $PO_4^{3-}$ - P | 1.0 ± 0.6 | 0.9 ± 0.7 | 1 | 3.62 | 0.50 | 0.52 |
| log(N:P) ratio | 3.1 ± 0.6 | 2.9 ± 0.7 | 1 | 3.96 | 0.06 | 0.82 |

*Where $DF_{num}$: numerotor's degrees of freedom, $DF_{den}$: denominator's degrees of freedom, F-value: F-test determination*

Table 3-3 shows the averages during Pre-treatment and Year 1 of all three soil bioavailable nutrients and N:P ratios in ambient and $eCO_2$ arrays. Although during the Pre-treatment periods, differences in the availability of all three soil nutrients and the N:P ratios were observed between the preassigned as ambient and $eCO_2$ arrays, none of these differences were found to be statistically significant. During Year 1 of $eCO_2$ enrichment, $eCO_2$ had a statistically significant effect only on $NO_3^-$-N availability, decreasing the $NO_3^-$-N availability by up to 37% under $eCO_2$ conditions. However, $NH_4^+$-N, $PO_4^{3-}$ and N:P ratios were statistically similar between ambient and $eCO_2$ conditions.

$T_s$ had a statistically significant effect on all three bioavailable nutrients ($NO_3^-$-N, $NH_4^+$-N, and $PO_4^{3-}$-P) as well as the N:P ratio (Table 3-4). VWC had a statistically significant

effect on N:P ratios, whereas no statistically significant effects were observed in the three bioavailable nutrients. The interactions between $eCO_2$ and the environmental variables (VWC and $T_s$) did not significantly affect the bioavailable nutrients or the N:P ratios.

***Table 3- 4:*** Summary of mixed-effects models of the environmental variables and their interactions with $eCO_2$ on bioavailable soil nutrients. Fixed factors are VWC, $T_s$, and their interactions with $eCO_2$. The degrees of freedom for each explanatory variable is 1. P-value < 0.1 is shown in bold.

| | | Predictors | | | |
|---|---|---|---|---|---|
| Response variables | | VWC | $T_s$ | $eCO_2$*VWC | $eCO_2$*$T_s$ |
| $NO_3^-$-N | F | 3.72 | 16.14 | 0.19 | 2.77 |
| | $Df_{den}$ | 20.69 | 36 | 20.69 | 36 |
| | P-value | 0.07 | **0.0003** | 0.67 | 0.10 |
| $NH_4^+$-N | F | 1.85 | 48.30 | 0.33 | 1.10 |
| | $Df_{den}$ | 36 | 32.75 | 36 | 32.75 |
| | P-value | 0.18 | **<0.001** | 0.57 | 0.30 |
| $PO_4^{3-}$-P | F | 3.77 | 25.16 | 0.93 | 2.64 |
| | $Df_{den}$ | 35.78 | 31.82 | 35.78 | 31.82 |
| | P-value | 0.06 | **<0.001** | 0.34 | 0.11 |
| N:P ratios | F | 5.53 | 10.96 | 0.55 | 2.51 |
| | $Df_{den}$ | 34.69 | 31.99 | 34.69 | 31.99 |
| | P-value | **0.02** | **0.002** | 0.46 | 0.12 |

## 3.5 Discussion

Soils at BIFoR FACE exhibited strong seasonal patterns in soil moisture, temperature, and respiration during approximately six months before the first $eCO_2$ enrichment as well as the first year of $eCO_2$ enrichment (Fig. 3-2). No significant differences were observed in soil respiration, temperature, moisture, and bioavailable soil nutrients

between ambient and $eCO_2$ arrays during the Pre-treatment period. However, following fumigation, $eCO_2$ had a positive effect on soil respiration but only when additional factors of soil temperature and soil moisture were taken into account (Table 3-2). Soil temperature had a stronger effect than soil moisture on both respiration and nutrient availability. $eCO_2$ also had a statistically significant negative effect on $NO_3^-$-N availability but affected neither $NH_4^+$-N nor $PO_4^{3-}$ availability.

### 3.5.1 Soil respiration and environmental drivers

The annual estimates of soil respiration during the first year of $eCO_2$ enrichment were 1,341±21 vs. 1,624±24 g C $m^{-2}$ $y^{-1}$ in ambient and $eCO_2$ arrays. These estimates are within the range of observations (500-2,100 g C $m^{-2}$ $y^{-1}$) reported for similar temperate forests (Campbell and Law, 2005). Soil respiration in the BIFoR FACE was observed to be at a higher range than other temperate forests; however, this is expected since mature forests have higher soil respiration than initiation young and old-growth forests. Our annual estimates are higher than the global mean value for deciduous broadleaf forests, ~850 g C $m^{-2}$ $y^{-1}$ (Xu and Shang, 2016) as well as other deciduous broadleaf forests (509-867 g C $m^{-2}$ $y^{-1}$ Hibbard et al., 2005; 663-913 g C $m^{-2}$ $y^{-1}$ Giasson et al., 2013). However, it is essential to note that the deciduous broadleaf forests included in Hibbard et al., (2005) were much younger than BIFoR FACE (31-75 years). In contrast, the Harvard forest soils in Giasson et al., (2013) are heavily influenced by prior land use (agricultural, pasture and woodlots use) and natural disturbances.

The seasonal patterns of soil respiration support our annual respiration estimates at BIFoR FACE, showing consistent higher soil respiration in $eCO_2$ arrays during Year 1 of $eCO_2$ enrichment (Fig. 3-2). These results are further supported by the mixed-effects models' outcome, where the $eCO_2$ interaction with soil temperature and the $eCO_2$ interaction with soil temperature and moisture were statistically significant.

Previous FACE experiments in forest ecosystems have observed different magnitudes of increase (Duke: 15.2%; FACTS-I: 29.4%, FACTS-II: 12.6% for *P. tremuloides* and 42.5% for *Betula/Populus*; POPFACE: 35.6% for *P. eur*, 49.5% for *P.alba* and 35.7% for *P. nigra*) or even a decrease (ORNL: 3%) in soil respiration rates during their first year of $eCO_2$ enrichment (King *et al.*, 2004; Bernhardt *et al.*, 2006). However, all these forest ecosystems were either plantations or young forests on disturbed soils. Moreover, in large open-top chambers studying the effects of $eCO_2$ in oak saplings, an increase of 22 and 37% was observed; however, the saplings were exposed to higher $eCO_2$ concentrations (Norby, 1996). EucFACE is the only FACE experiment conducted in mature woodland. The observed increase in annual soil respiration observed was approximately 7% (Drake *et al.*, 2018), similar to that observed at BIFoR FACE.

The models used for the soil respiration estimates were the $eCO_2$ and soil temperature interaction for the Pre-treatment period and the triple interaction between $eCO_2$, soil temperature, and moisture for Year 1. Both models predicted 64 and 63% of the variability (Table 3-2). Our modelling estimates are defensible since the correlation between predicted and observed soil respiration values was statistically significant in ambient and $eCO_2$ arrays. Two plausible reasons explain the relatively moderate performance observed in our modelling approach. Although soil moisture is an essential regulator of soil respiration, our study site is a mesic ecosystem, where soil moisture was not strongly limiting soil respiration rates most of the time. Secondly, although including soil moisture as a predictive variable may increase the model's predictive power, albeit it increases the prevalence of unreasonable fits, especially as the gap fraction between measurements increases (Gomez-Casanovas *et al.*, 2013). Such modelling approaches can perform better under small data-gap fractions, and

the unreasonable fits increase as the gap fractions increase. However, it is essential to note that the model's performance decreases when there is a plethora of temporal variability with limited spatial variability. Although our gap fraction was approximately 13%, it is essential to note that the gap fraction was spatial due to the experimental design and instrumental limitations (one ambient and one $eCO_2$ measured per hour for two weeks).

$eCO_2$ was observed to alter soil respiration response to the combined soil moisture and soil temperature effects. In ambient arrays, high respiration was observed under high temperature and low soil moisture. However, in eCO2 arrays, despite soil temperature being the primary driver of soil respiration, our analysis highlights soil moisture importance (Fig. 3-4, Table 3-2). When soil moisture was included in our mixed-model analysis, the model's predictive capability increased by 2% (61 vs. 63% predictive capacity, respectively).

Soil moisture did not follow soil temperature and soil respiration's aforementioned seasonal pattern (Fig. 3-2). Soil moisture followed a seasonal course which is characteristic of temperate forests, with high levels in late autumn, winter, and spring, and lower levels in summer and early autumn (Davidson, Belk and Boone, 1998; Savage and Davidson, 2001; DeForest *et al.*, 2006; Turner and Henry, 2010; Contosta, Frey and Cooper, 2011). An optimum water content where soil respiration rates peaked (~ 10% volumetric water content) was observed (see section *3.8 Supplementary Material Fig. S3-1*), but as the soil moisture increased further, soil respiration was suppressed. Our findings are in agreement with previous studies, which show that intermediate water content is commonly found to be optimum for soil respiration (Davidson, Belk and Boone, 1998; Davidson *et al.*, 2000; Drewitt *et al.*, 2002; Davidson and Janssens, 2006; Giasson *et al.*, 2013). High water content can

reduce root growth and function, primarily because of decreased oxygen availability in the soil pores (Kutsch, Bahn and Heinemeyer, 2010). On the other hand, dry soil conditions decrease the root water content, followed by a decrease in root cell turgor pressure. Consequently, since root cell turgor pressure is essential for plant growth, this will reduce metabolic activity and, finally, decrease soil respiration rates (Kutsch, Bahn and Heinemeyer, 2010).

Soil respiration seasonal cycles followed soil temperature's seasonal cycles, with soil respiration being highest in summer and lowest in winter (Fig. 3-2). Soil respiration peaks during the growing season when the soil temperature is high, and root exudates of labile C are likely to be abundant. In contrast, soil respiration tends to reach its lowest levels during winter when the soil temperature is low and labile C is limited (Contosta, Frey and Cooper, 2011). The observed pattern agrees with previous studies in temperate forest ecosystems (Davidson, Belk and Boone, 1998; Jaeger. C.H. III *et al.*, 1999; Bohlen *et al.*, 2001; Hogberg *et al.*, 2001; Knoepp and Swank, 2002; Bowden *et al.*, 2004; Mo *et al.*, 2005; DeForest *et al.*, 2006; Contosta, Frey and Cooper, 2011).

Temperature sensitivity $Q_{10}$ was 3.08 in ambient vs. 2.64 in $eCO_2$ arrays during Year 1 at BIFoR FACE, lower than other deciduous forests (3.83; Giasson *et al.*, 2013) and the global average (3.46; Bond-Lamberty and Thomson, 2010). Moreover, the lower $Q_{10}$ in $eCO_2$ arrays indicated that soil respiration under $eCO_2$ conditions was less sensitive to soil temperature. However, $Q_{10}$ has been criticized since it is calculated via seasonal variations in temperature (see also *Chapter 2*), it is challenging to distinguish the temperature effect from other seasonal factors, such as carbon allocation to roots, solar radiation, and soil moisture (Davidson, Janssens and Lou, 2006; Almagro *et al.*, 2009; Jia *et al.*, 2013; Han *et al.*, 2014; Y. Wang *et al.*, 2014),

and $Q_{10}$ has been observed to decrease as soil moisture increased in forest soils (Guntiñas *et al.*, 2013), thus distinguishing whether the $Q_{10}$ decrease was due to $eCO_2$ or due to increased soil moisture under $eCO_2$ conditions, was challenging.

Although investigating soil responses to environmental factors individually might provide a simpler approach, *in situ,* the environmental factors interact in a more complex and dynamic way with soil respiration. However, in either very low or very high soil temperatures, soil moisture's effect on soil respiration was almost negligible, suggesting that soil temperature is the primary driver of soil respiration. It is commonly observed in temperate ecosystems that soil temperature is the primary driver of soil respiration (Lloyd and Taylor, 1994; Giasson *et al.*, 2013; Finzi *et al.*, 2014), and soil moisture becomes a controlling factor only at very high or very low levels (Lellei-Kovács *et al.*, 2011).

Soil respiration responded rapidly to rain events. The magnitude of the soil respiration increase depended on the antecedent soil conditions, with greater soil respiration effects after a prolonged dry period. This observation is in agreement with other studies (Unger *et al.*, 2010). The rainfall effect was greater if it was after a prolonged dry and warm period, almost doubling the soil respiration, even if the rainfall event was approximately 1 mm. However, no further increase in soil respiration was observed when the rainfall event was after a period of high soil moisture, even under high precipitation event (> 3mm). The pulses of soil respiration, often referred to as the Birch Effect (Birch, 1964), as a response to rainfall events can be explained due to physical $CO_2$ displacement from soil pore spaces (Liu *et al.*, 2002; Maier *et al.*, 2010). Moreover, after a rainfall event, previously unavailable C substrates that had accumulated when soils were dry may become available to microbes due to an increase in soil moisture (Denef, Six, Bossuyt, *et al.*, 2001; Denef, Six, Paustian, *et*

*al.*, 2001; Six *et al.*, 2004; Göransson *et al.*, 2013). Another plausible hypothesis for the soil respiration pulse observed after a rainfall event is the osmolytes used by microbes to resist desiccation during dry conditions being metabolized (Fierer and Schimel, 2003; Xiang *et al.*, 2008; Williams and Xia, 2009) or due to microbial cell lysis due to excess turgor pressure, leading to a release of carbohydrates and other labile C solutes, and their subsequent consumption by surviving microbes (Xu, Baldocchi and Tang, 2004; Schimel, Balser and Wallenstein, 2007; Borken and Matzner, 2009; Warren, 2014).

According to the ecosystem's mass balance, the observed increase in soil $CO_2$ effluxes must be equated by additional C inputs, such as litterfall or the C loss from soil pools (Giardina and Ryan, 2002). It is already well established that alterations in C inputs to soils, such as litterfall, can explain soil respiration responses to $eCO_2$ (Drake *et al.*, 2011). However, the immediate soil respiration responses observed within days after the $eCO_2$ enrichment, combined with the period that the $eCO_2$ enrichment started (deciduous trees do not shed leaves early on the growing season), imply that the soil $CO_2$ efflux stimulation cannot be attributed to increased litterfall inputs in the $eCO_2$ arrays. Therefore, we suggest that the prompt and sustained soil respiration response to $eCO_2$ was due to increased C allocation belowground to roots and mycorrhizae.

### 3.5.2 Response of bioavailable nutrients to $eCO_2$

During the first year of $eCO_2$ enrichment at BIFoR FACE, $eCO_2$ had a statistically significant effect on $NO_3^-$-N availability, decreasing the availability by 27%, on average, in $eCO_2$ arrays (Fig. 3-6 and Table 3-3-3). In contrast, $eCO_2$ had no statistically significant effect on $NH_4^+$-N and $PO_4^{3-}$-P availability, nor on N:P ratios. All three soil nutrients and N:P ratios exhibited strong seasonal patterns, with their availability

higher in summer and lower in winter. Soil temperature had a statistically significant effect on all three nutrients and N:P ratios; however, soil moisture had a statistically significant effect on N:P ratios (Table 3-4). Neither soil temperature nor soil moisture's interaction with $eCO_2$ was statistically significant.

All three soil nutrients and N:P ratios exhibited the same response pattern towards a combined effect of soil moisture, soil temperature, and monthly cumulative rainfall (see section 3.8 Supplementary material Fig. S3-4). Nutrient availability increased as soil temperature and cumulative precipitation increased while soil moisture decreased. The higher the soil temperature and rainfall for the same soil moisture level, the higher the nutrient availability. However, the higher the precipitation for the same soil temperature level, the higher the availability, while soil moisture's effect appeared almost negligible in both ambient and $eCO_2$ arrays.

The effect of $eCO_2$ on $NO_3^-$-N availability is highly uncertain since it has been observed to increase, decrease or have no significant impact (Bassirirad *et al.*, 1996; BassiriRad *et al.*, 1997; BassiriRad *et al.*, 1997; Hasegawa, Macdonald and Power, 2016). Soil $NO_3^-$-N availability under $eCO_2$ could be affected due to N mineralization and microbial immobilization (Díaz *et al.*, 1993; Hungate, 1999) or dinitrogen fixation (Hartwig *et al.*, 1996). Moreover, it could be affected due to leaching or plant uptake pathways such as total plant N pools (Owensby, Coyne and Auen, 1993), root uptake kinetics (Bassirirad *et al.*, 1996), root length density, and root system size (Newton *et al.*, 1996).

Uptake and assimilation of both N forms are essential for plant growth and require the expenditure of energy. It is well documented that $NO_3^-$-N requires considerably more energy for uptake and assimilation than $NH_4^+$-N because $NO_3^-$-N must be metabolically

reduced to ammonia before it can be assimilated (Haynes and Goh, 1978; Blacquière, Hofstra and Stulen, 1987; Glass and Siddiqi, 1995). Thus, the distinct root preference for $NH_4^+$-N uptake is especially pronounced in tree species, possibly as an adaptation mechanism to forest soils that are relatively low on $NO_3^-$-N content (Bassirirad *et al.*, 1996). Specifically for oaks, it has been observed that, during the seedling stage, there is a slight $NH_4^+$-N preference (Stadler, Gebauer and Schulze, 1993). However, a shift towards $NO_3^-$-N preference has been observed in sites where nitrification is more rapid (Singh, Singh and Gupta, 2014). However, studies have shown increased uptake $NO_3^-$-N capacity without changes in root N concentration, indicating that the increased uptake was due to increased carbohydrates allocation to the roots (Bassirirad *et al.*, 1996). Thus, $NO_3^-$-N uptake and utilization may be disproportionally enhanced by $eCO_2$ compared to $NH_4^+$ -N due to higher energy root status (BassiriRad *et al.*, 1997).

Another pathway explaining the decrease in $NO_3^-$-N availability in $eCO_2$ arrays is uptake by ectomycorrhizal hyphae. Mycorrhizal hyphae form symbiotic relationships with the plants exchanging nitrogen and phosphorus for organic carbon. There is speculation that forests with ectomycorrhizal associations operate in an organic nutrient economy due to the decelerated litter decomposition rates, which result in greater amounts of soil organic matter being accumulated (Phillips, Brzostek and Midgley, 2013). Hence, ectomycorrhizal hyphae can acquire more nitrogen from soil organic matter, providing more available N to the plants. Certain ectomycorrhizal hyphae species have been observed to grow better on $NO_3^-$ than $NH_4^+$ (Aouadj et al., 2000; Scheromm et al., 1990). Moreover, many ectomycorrhizal hyphae have been observed to acquire $NO_3^-$-N and metabolize it as an N source (Nygren, 2008). However, there are limited studies on ectomycorrhizal hyphae and $eCO_2$, but a few of them report inconsistencies on ectomycorrhizal hyphae responses under $eCO_2$

regarding both colonization (Garcia *et al.*, 2008; Wang *et al.*, 2015) and biomass (Gutknecht, Field and Balser, 2012).

Microbial processes, such as nitrification, immobilization, and denitrification, may also explain the observed decrease in $NO_3^-$-N availability in $eCO_2$ arrays could be through microbial. Nitrification is a step-wise process and is two different autotrophic bacterial groups perform each step. Nitrification is affected by various environmental factors, such as oxygen, temperature, moisture, the abundance of $NH_4^+$, population, diversity of nitrifying microorganisms, and substrates' availability to those organisms (Yuan *et al.*, 2005). Nitrification is observed to be inhibited at higher soil moisture ranges (>60%); however, soil moisture at BIFoR FACE was observed to be consistently lower than 30%. Both soil temperature and moisture did not reach levels that could halt nitrification during $eCO_2$ enrichment. The NH4+ availability was similar under both ambient conditions and eCO2 conditions; thus the inhibition of nitrification a plausible explanation for the observed decrease in $NO_3^-$-N availability is unlikely. In the short term, no $eCO_2$ effect has been observed on nitrification (Rütting and Andresen, 2015) and autotrophic nitrifiers (Hu *et al.*, 2016). Although gross nitrification has been observed to decrease in FACE experiments, the datasets are small in size; thus, caution must be taken when interpreting these results (Rütting and Andresen, 2015). Thus the observed decrease in $NO_3^-$-N availability could be attributed to increased microbial and plant uptake under $eCO_2$ enrichment.

It has been observed that in the short term, microbes are responsible for substantially faster initial uptake of all N forms and, more specifically, a higher capacity for $NO_3^-$-N (Kuzyakov and Xu, 2013). Although the overall microbial biomass increased only by 7% under $eCO_2$ conditions, based on a meta-analysis by de Graaff *et al.*, (2006), $NO_3^-$ immobilization has increased under $eCO_2$ conditions (Zak *et al.*, 2000). However, it is

still unclear the effect of $eCO_2$ on microbial processes, since the findings in the literature regarding microbial biomass, N mineralization, and microbial immobilization under $eCO_2$ conditions report both large increases and decreases in all three processes mentioned above (Hungate, 1999; Zak *et al.*, 2000).

Another plausible explanation for the decrease in $NO_3^-$-N availability under $eCO_2$ is increased nitrous oxide ($N_2O$) emissions from soil. Although in the short-term, $eCO_2$ enrichment did not affect nitrate-reducing organisms (Marhan *et al.*, 2011), $eCO_2$ can enhance the $N_2O$ emissions from 27 up to 50% (Ineson, Coward and Hartwig, 1998; Kee Lam *et al.*, 2010). $N_2O$ emissions can be enhanced under $eCO_2$ by increased root-derived available C, thus providing energy for denitrification, stimulating root biomass, and exudates for denitrifiers (Ineson, Coward and Hartwig, 1998; Kettunen *et al.*, 2007). Such increases in $N_2O$ are considered to offset $CO_2$ sequestration by more than 70% (Liu and Greaver, 2009), significantly impacting the terrestrial C sink.

Studies have shown that $NO_3^-$-N availability was severely affected under $eCO_2$ due to increased leaching, and the effect was exacerbated under the combination of $eCO_2$ and N addition (Liu *et al.*, 2008). Temperate forests are predicted to face a gradual decline in their stability as the atmospheric N deposition increases, leading to nitrogen saturation (Schulze, 1989; MacDonald *et al.*, 2002). These N-saturated forests are predicted to undergo an initial rise in soil N availability, mainly in $NO_3^-$-N, followed by substantial leaching. Thus, considerable leaching might accelerate nutrient limitations in ecosystems affected by chronic N deposition, subsequently affecting the amount of C sequestered and stored in these ecosystems and leading to more significant climate change impacts.

It has been generally observed that net N mineralization rates increase with temperature (Rustad *et al.*, 2001; Shaw and Harte, 2001; Melillo *et al.*, 2002; Wang *et al.*, 2006), thus higher rates of $NH_4^+$-N are bioavailable as the growing season progresses. Although soil moisture can significantly affect net N mineralization (Sierra, 1997; Paul *et al.*, 2003), the maximum net N mineralization rate occurs when soil moisture is near field water holding capacity (Stanford and Epstein, 1974). In contrast, nitrification is generally accepted to be halted at high soil moisture conditions. In contrast, the effect of soil temperature on nitrification follows a bell-shaped curve with optimum conditions at 30-35 °C (Sahrawat, 2008). Nonetheless, our dataset exceeded the optimum conditions neither for soil temperature nor for soil moisture.

Furthermore, increased soil temperature and soil moisture have been observed to increase $PO_4^{3-}$-P availability (Li, Mahler and Everson, 1990). N mineralization is a more robust process than nitrification. A plethora of bacteria, fungi, and free-living organisms contribute to the decomposition of organic matter and, subsequently, N mineralization. Moreover, decomposition takes place both under aerobic and anaerobic conditions, whereas nitrification requires well-aerated soils.

Substantial enhancement has been observed on N mineralization rates under $eCO_2$ conditions; the literature on FACE experiments is inconclusive regarding the $eCO_2$ effect on $NH_4^+$-N availability and consumption (Rütting and Andresen, 2015). Our results contradict the results of other studies of $eCO_2$ in mature forests that have observed an increase in $NH_4^+$-N availability (Hasegawa, Macdonald and Power, 2016; Schleppi, Körner and Klein, 2019). Moreover, it has been reported that plants' demand for phosphorus increases due to growth stimulation under $eCO_2$ conditions (Gentile *et al.*, 2012; Zhang *et al.*, 2014). Moreover, the symbiotic relationship between mycorrhizal fungi and host plants can be stimulated under $eCO_2$ since fungi will

increase their P foraging activities in exchange for the increased root carbohydrate supply (Kiers *et al.*, 2011). Mycorrhizal fungi have been reported to enlarge their network under $eCO_2$ conditions; thus, nutrient absorption increases (Staddon, Gregersen and Jakobsen, 2004). Moreover, increased P immobilization by microbes under $eCO_2$ conditions has also been reported (Jin *et al.*, 2014). However, literature P availability in forest ecosystems under $eCO_2$ is limited and conflicting, since both no effects (Johnson *et al.*, 2004)or positive effects (Dijkstra *et al.*, 2012; Hasegawa, Macdonald and Power, 2016) have been reported.

### 3.5.3 Interactions of carbon and nutrient cycling to $eCO_2$

Environmental variables such as soil moisture, soil temperature, precipitation, nutrient demand, and supply are key processes contributing to the observed soil respiration. Other factors such as plant growth (De Vries, Brunsting and Van Laar, 1974), plant metabolism (Wit, Brouwer and Vries, 1970; Wieser and Bahn, 2004), root production and growth (Högberg and Read, 2006; Kutsch, Bahn and Heinemeyer, 2010; An *et al.*, 2017) root system micro-environment (Lambers, Atkin and Millenaar, 2002; Heinemeyer *et al.*, 2007; Kutsch, Bahn and Heinemeyer, 2010), and free-living mycorrhizosphere biota (Raich and Tufekcioglu, 2000; Mitra *et al.*, 2014; Brady and Weil, 2017), are crucial contributors to soil respiration. However, from an ecosystemic perspective, carbon and nutrient cycles are interlinked and interact with each other.

This study observed a prompt and sustained response of soil respiration to $eCO_2$ combined with soil temperature and moisture. Given the data available to this study, we hypothesised that the increased soil respiration response under $eCO_2$ is plausible to be attributed to increased carbon allocation belowground to roots, mycorrhiza

hyphae, and free-living microbes. We also observed a significant decrease in $NO_3^-$-N availability which was obvious just after a month of $eCO_2$ enrichment and was sustained until the end of the first year of $eCO_2$ enrichment. Interestingly, no differences were observed in $NH_4^+$-N availability in ambient and $eCO_2$ arrays, and the environmental conditions recorded at BIFoR FACE did not reach limiting levels for the nitrification process. Moreover, given that this was an initial short-term response, we can reject the hypothesis that the observed decrease in $NO_3^-$-N availability under $eCO_2$ was due to increased $N_2O$ emissions.

Ergo the suggested pathway for our observed responses during the first year of $eCO_2$ enrichment at BIFoR FACE is increased carbon allocation through roots to the soil, thus increasing the carbon substrate available to microorganisms. Soil microorganisms and plants utilised the extra carbon, and in order to meet their metabolic demands, they increased their nutrient uptake in the form of $NO_3^-$-N.

### 3.5.4 Future implications

Expectations are that maybe mature forests respond differently to $eCO_2$ enrichment than young forests due to different developmental C needs and internal-plant C allocations (Körner, 2006). During the early tree stages, more effort is allocated to increasing height and foliage development. During later stages in tree development, stem growth and root development may become the priority for resource allocation (Peichl and Arain, 2007). Moreover, the magnitude of the $eCO_2$ effect on mature forests may be constrained by nutrient availability. Mature and old-growth forests are operating under a self-supporting system in terms of nutrition, with nutrient availability rates controlled by microbial activity (Ingestad, 1982). If the nutrient demand rates exceed the nutrient availability rates under $eCO_2$, the possibility of increased carbon

allocation belowground for an attempt to advance foraging nutrients may be restricted in mature forest ecosystems.

Although this study focused only on the first year of $eCO_2$ enrichment at a mature temperate woodland and continuous observation is essential since different processes have different timescales. Short-term responses are not always sustained, and they tend to disappear or alter after a few years (Drake *et al.*, 2018; Ochoa-Hueso, Piñeiro and Power, 2019). However, if the observed responses in this study are sustained, the ecosystem can undergo a progressive nitrogen limitation (PNL) (Finzi *et al.*, 2006). PNL under $eCO_2$ can occur as a swift mineral N immobilization by plant uptake, leading to mineral N soil depletion from the labile pools, resulting in slower N mineralization rates and a subsequent decrease in N availability (Luo *et al.*, 2004). Moreover, PNL can occur due to an increased microbial N demand due to the increased microbial C availability, leading to increased N immobilization by microorganisms, and decreased N availability to plants (Luo *et al.*, 2004). If mature forests are faced with nutrient limitations under $eCO_2$, the net primary production will not respond anymore to $eCO_2$. This is crucial since, for the past 20 years, temperate forests have played an essential role as $CO_2$ sinks. Thus the forests' capacity to uptake and store carbon long-term might be compromised, pausing greater uncertainties to climate change.

## 3.6 Conclusions

The purpose of this study was to monitor the soil respiration and the bioavailability of soil nutrients in a mature temperate woodland during its first year of $eCO_2$ enrichment to +150 ppm above ambient conditions. This is the first time that belowground responses of soil $eCO_2$ fluxes and nutrient dynamics of mature temperate woodland are under high-resolution scrutiny, both temporally and spatially. Moreover, this study

provided the advantage to study the responses of the woodland under the 2050 atmospheric $CO_2$ scenario as a coetaneous event.

Temperate deciduous forest ecosystems exhibit strong seasonality in their physiology, environmental variables, and, subsequently, their biochemical responses. During winter, deciduous forests do not have leaves, soil moisture is high, and soil temperature is low. Accordingly, soil respiration rates and soil nutrient bioavailability are low. Thus, seasonality had a significant effect on both soil respiration rates and soil nutrient bioavailability.

Soil temperature had a statistically significant effect on all three bioavailable soil nutrients ($NO_3^-$-N, $NH_4^+$-N, and $PO_4^{3-}$-P), and all nutrients' availability increased as soil temperature increased. However, soil moisture had a negative effect on $NH_4^+$-N and $PO_4^{3-}$-P availability, with the effect being more prominent for $NH_4^+$-N availability. $NO_3^-$-N availability responded negatively to soil moisture under low water content, albeit $NO_3^-$-N availability increased as the soil moisture increased. Soil moisture's effect on all three nutrients was not statistically significant. Both soil moisture and soil temperature had a statistically significant effect on N:P ratios. Soil temperature had a negative impact on N:P ratios, while soil moisture had almost no effect on N:P ratios in low and medium water content. However, as soil moisture increased to high water content, the effect on N:P ratios was negative.

Addressing the research questions posed at the beginning, we suggest the following.

- Does $eCO_2$ affect soil respiration, soil moisture, and soil temperature on a mature oak woodland?

Although soil respiration was higher in $eCO_2$ arrays (up to 46% increase in monthly averages), $eCO_2$ had no statistically significant effect on soil respiration. The annual

soil respiration was approximately 21.1% higher in $eCO_2$ arrays, feeding back to the atmosphere and an additional 283 g C $m^{-2}$ $y^{-1}$. Per contra, although soil moisture and soil temperature were slightly higher under $eCO_2$ conditions, they were statistically similar between ambient and $eCO_2$ conditions. Moreover, soil respiration rain pulses were more prominent under $eCO_2$ conditions than under ambient conditions.

The interactions between $eCO_2$, soil moisture, and soil temperature had a statistically significant effect on soil respiration; thus, soil respiration responses to the environmental variables were altered under $eCO_2$ conditions. The effect of soil temperature on soil respiration was more pronounced under $eCO_2$ conditions. The effect of soil moisture was more prominent under eCO2 conditions, and the tipping point in which soil moisture's effect switches from positive to negative was shifted to higher water content. Thus, the highest respiration rates under $eCO_2$ conditions were reported under high soil temperatures regardless of the water content.

- How do bioavailable soil nutrients respond to $eCO_2$?

$eCO_2$ had a statistically significant effect on $NO_3^-$-N availability, decreasing the seasonal availability by 27% on average. No statistically significant $eCO_2$ effect was observed on $NH_4^+$-N and $PO_4^{3-}$-P availability nor on the N:P ratios. $eCO_2$ interactions with both soil moisture and soil temperature were statistically similar under ambient and $eCO_2$ conditions. Although soil moisture, soil temperature, and rainfall had an interactive and dynamic effect on nutrient availability, it appeared that soil temperature and cumulative monthly precipitation were the main drivers of soil nutrient availability.

Combining the overall belowground responses observed during the first year of $eCO_2$ enrichment at BIFoR FACE, we suggest that those responses are most likely attributed to increased rhizosphere activity under $eCO_2$ conditions due to an increase in

belowground carbon allocation. The major environmental drivers for such belowground responses are soil moisture and soil temperature, which were statistically similar under ambient and $eCO_2$ conditions. Thus, these responses are more likely to be driven by an increased photosynthate transport belowground, increasing the root respiration and the mycorrhizal and microbial respiration due to increased carbon supply. For the increased soil respiration response to be accommodated, the $NO_3^-$ demand increased. It could either be attributed either to increased plant demand and subsequent uptake through the mycorrhiza's symbiotic relationship or to increased microbial activity and subsequent immobilization. Both pathways can affect the ecosystem's long-term responses differently, and ultimately the forest's potential to sequester more carbon.

## 3.7 References


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## 3.8 Supplementary material

The data presented in Fig. S3-1 are only during the $eCO_2$ enrichment period (April – October 2017), to assess both ambient and $eCO_2$ conditions and exclude the winter period (low $T_s$ and high VWC) in an attempt to isolate each drivers effect. A positive effect of VWC on $R_s$ rates (Fig. 3a) was identified under low VWC levels (<10%) under ambient conditions, followed by a plateau response until ~ 14%. As the VWC levels increased further than those levels, the effect of VWC on $R_s$ was negative. Although it appears as the VWC in higher levels halted $R_s$, the VWC levels were not that high as to prohibit $R_s$, thus this response suggests an interaction with $T_s$. Under $eCO_2$ conditions, the $R_s$ responses followed the same pattern as in ambient conditions, but the $R_s$ rates were higher. Moreover, the plateau response in the medium VWC levels under ambient conditions was not observed under $eCO_2$ conditions. Instead, VWC had a positive effect on $R_s$ rates under $eCO_2$ conditions until ~15% water content followed by negative feedback as the VWC levels increase. Antithetically, the effect of $T_s$ on $R_s$,

under ambient conditions, was positive across the whole range of $T_s$ during Year 1(Fig. S3-1b). The $R_s$ response on $T_s$ under $eCO_2$ conditions was the same as in ambient conditions, but the $R_s$ rates were higher. However, as $T_s$ increased above 15 °C it had a negative effect on $R_s$ rates, suggesting an interaction with VWC.

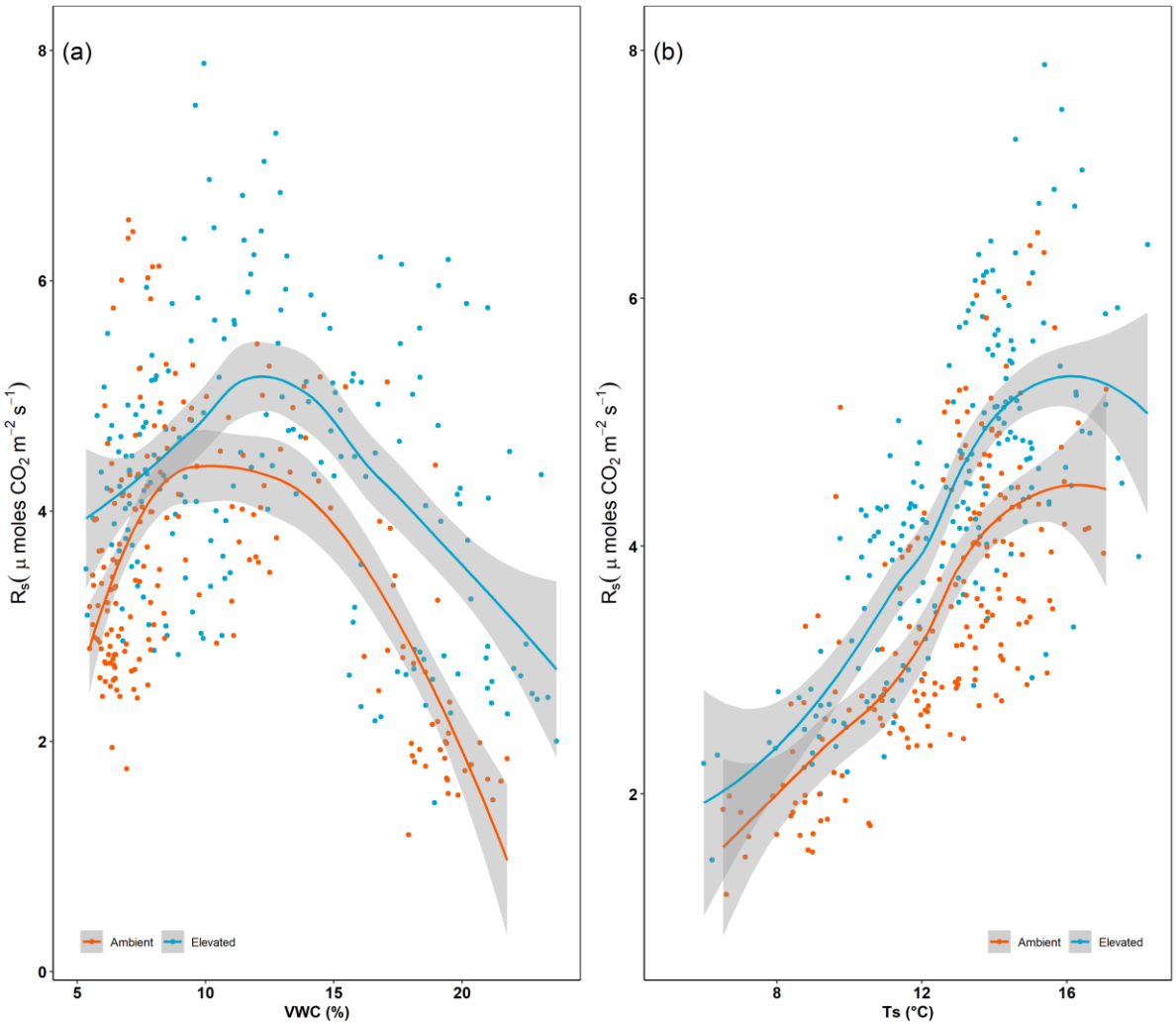


***Figure S3- 1:*** *$R_s$ response (LOESS) on VWC (a) and $T_s$ (b) in both $eCO_2$ and ambient conditions during Year 1. The blue solid points represent the $eCO_2$ arrays, while the orange solid points represent the ambient arrays.*

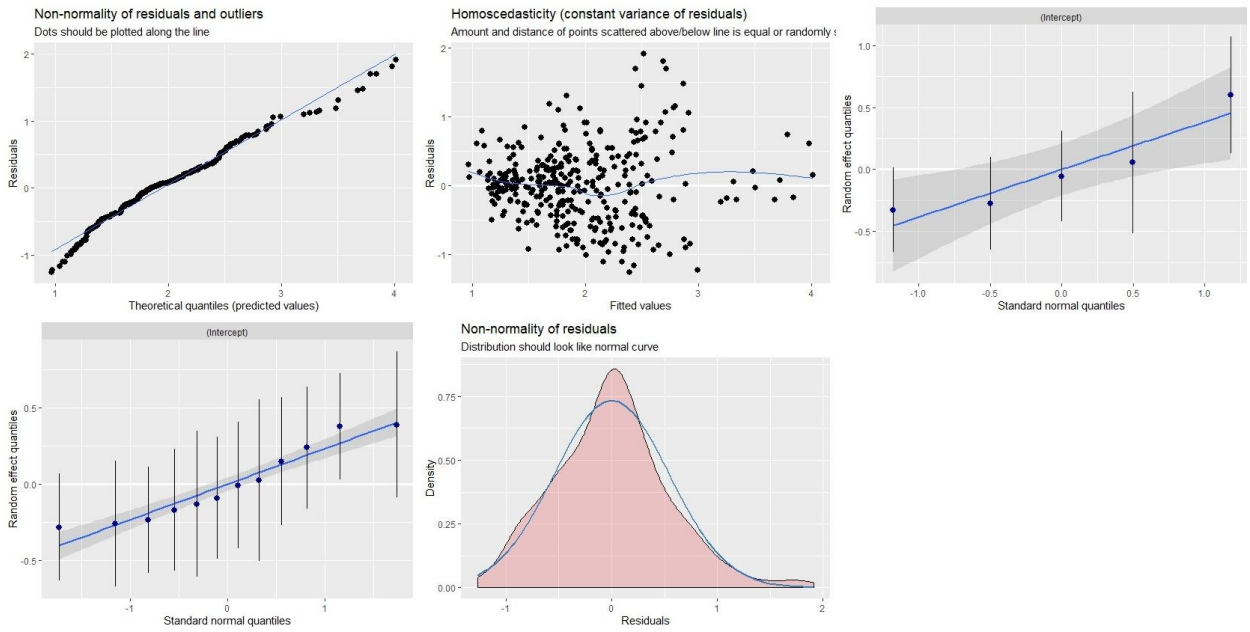


***Figure S3- 2:*** *Model validation for estimating soil respiration during Pre-treatment for homoscedasticity, normality of residuals, and outliers and random effects.*

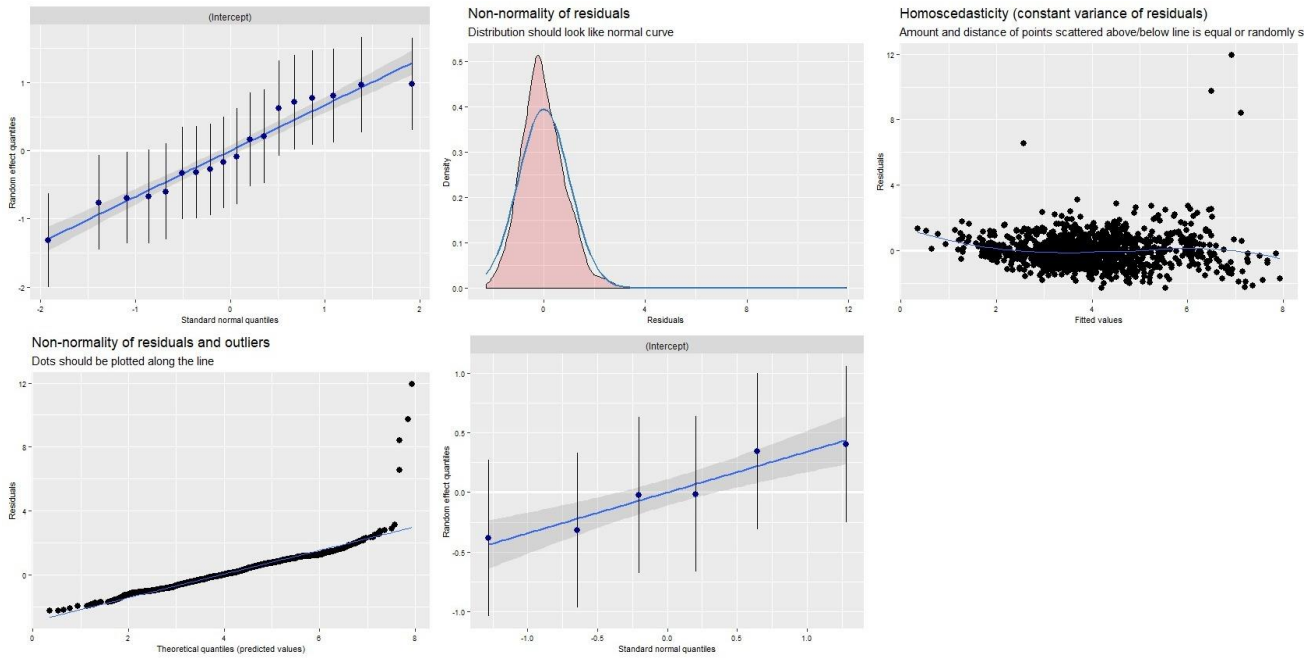


***Figure S3- 3:*** *Model validation for estimating soil respiration during Year 1 for homoscedasticity, normality of residuals, and outliers and random effects.*

Table S3-1 shows the mean regression coefficients for the best-fitted models for Pre-treatment and Year 1.

***Table S3- 1:*** *Fixed effects parameter estimates (coefficients) for the best-fitted model for Pre-treatment and Year 1.*

| | Estimate | Std. Error | t-value |
|---|---|---|---|
| *Pre-treatment* | | | |
| Intercept | 1.15 | 0.66 | 1.74 |
| $eCO_2$ | 0.33 | 0.74 | 0.45 |
| $T_s$ | 0.05 | 0.07 | 0.67 |
| $eCO_2$:$T_s$ | 0.10 | 0.07 | 1.37 |
| *Year 1* | | | |
| Intercept | 0.65 | 0.54 | -1.19 |
| $eCO_2$ | 2.86 | 0.79 | 3.60 |
| VWC | 0.003 | 0.002 | 1.55 |
| $T_s$ | 0.3525 | 0.03 | 11.90 |
| $eCO_2$:VWC | -0.01 | 0.002 | -6.14 |
| $eCO_2$:$T_s$ | -0.18 | 0.04 | -4.14 |
| VWC: $T_s$ | -0.0003 | 0.0002 | -1.79 |
| $eCO_2$* VWC* $T_s$ | 0.001 | 0.0002 | 5.92 |

Fig. S3-4 shows the soil bioavailable nutrient and N:P ratios responses to VWC, $T_s$, and precipitation at BIFoR FACE during Year 1. All three nutrients ($NO_3^-$-N, $NH_4^+$-N, and $PO_4^{3-}$-P), as well as the N:P ratios, exhibited the same pattern in response to all three environmental variables, under both ambient and $eCO_2$ conditions. The highest nutrient availability was observed under low VWC, high $T_s$, and high precipitation, whereas the lowest nutrient availability was observed under high VWC, low $T_s$, and low precipitation. Moreover, under the same $T_s$ and precipitation, the nutrient availability regardless of the VWC level, suggesting that the main drivers for nutrient availability were $T_s$ and precipitation.

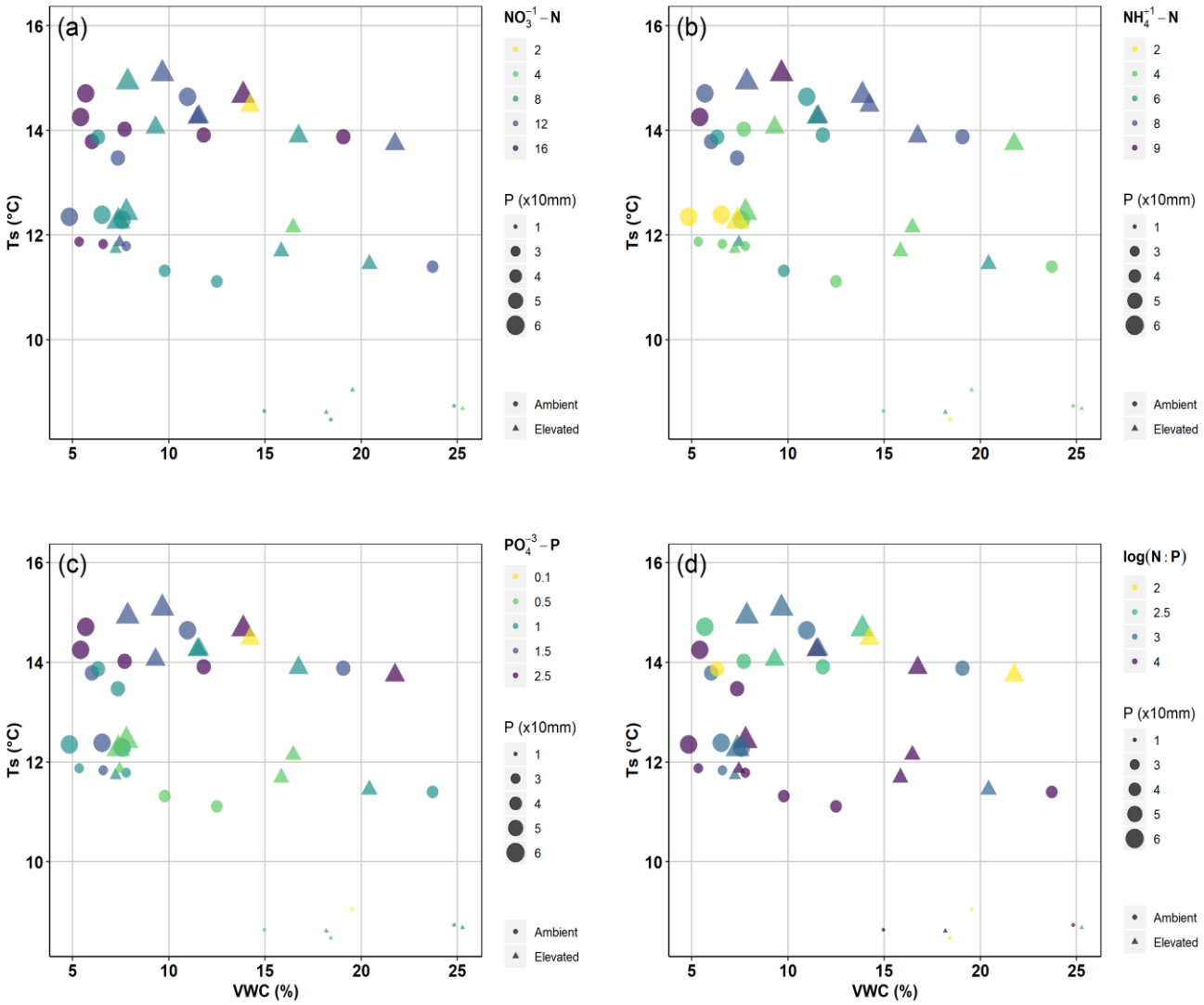


***Figure S3- 4:*** (a) $NO_3^-$-N, (b) $NH_4^+$-N, (c) $PO_4^{3-}$P availability, and (d) N:P ratios in correlation with VWC, $T_s$, and P at BIFoR FACE during Year 1 of $eCO_2$ enrichment. The triangles reflect the $eCO_2$ conditions while the circles reflect the ambient conditions. The colour luminance reflects the monthly cumulative precipitation in *10 mm, which increases as the precipitation increases. The size of the shapes reflects the availability of soil nutrients and N:P ratios, which increase as the availability increases.

# Chapter 4: Dominance of extreme events over elevated $CO_2$: the responses of forest belowground processes in a Free Air $CO_2$ Enrichment experiment

## 4.1 Abstract

Elevated $CO_2$ ($eCO_2$) and extreme events (such as heatwaves and droughts) are closely associated with climate change, potentially impacting belowground processes such as soil respiration and nutrient cycling. However, the relative importance of the $eCO_2$ or extreme events remains unknown. Here, we evaluate the impact of extreme events (extreme climatic and biological events) on belowground processes during a Free Air $CO_2$ Enrichment (FACE) at a mature temperate woodland. The drought had a severe effect on soil respiration, suppressing soil respiration by 47 and 40% in ambient and $eCO_2$ arrays, respectively, compared with the previous year. $eCO_2$ dampened the extreme event's impact by increasing soil respiration by ~36% in $eCO_2$ arrays. A winter moth outbreak led to a short-lived manifold increase in soil nutrient availability during autumn for all three nutrients, with the effect being more prominent under $eCO_2$.

## 4.2 Introduction

Anthropogenic emissions of carbon dioxide ($CO_2$) are the highest ever recorded, with over a twofold increase in the past 170 years (IPCC, 2014a). Concurrently, both the land surface temperature and precipitation are documented to follow ascending patterns over the same period, with the effect being more prominent in the Northern

Hemisphere (IPCC, 2014b). Future projections predict different magnitudes, albeit in agreement, of the continuous increase in atmospheric $CO_2$ concentration and its relationships with land surface temperature and precipitation to be directly correlated (Collins *et al.*, 2013). Increases in the frequency and/or intensity of extreme weather, climate, and biological events (EE) observed in the recent decades have been linked to the changes mentioned above in the climate (Classen *et al.*, 2005; Easterling *et al.*, 2017). These extreme-event stressors and/or their interactions increase the risks of severe or irreversible impacts on natural ecosystems since they are modifying hydrological systems, altering populations' life cycles and activities, and shifting species migration (IPCC, 2014b). Therefore, EE can dramatically influence the regional C cycle by altering the structure, composition, and functioning of terrestrial ecosystems and possibly shifting a current C sink to a C source (Frank *et al.*, 2015).

However, the rate of atmospheric $CO_2$ increase is not only conditional on the anthropogenic emissions (UNFCCC, 2005) but also on the ecosystems' efficiency to uptake carbon (C) and store it (Friedlingstein *et al.*, 2006, 2014). Forest ecosystems are immensely significant for the C cycle since it is estimated that ~ 860 ± 60 Pg C are currently being stored in the world's forests (Pan *et al.*, 2011), with the Northern temperate forests being the primary regulators (Schimel, Stephens and Fisher, 2015). Moreover, forests are expected to endure the most significant net effects of climate change on the terrestrial C balance than other ecosystems (Frank *et al.*, 2015). EE and their impact on the global C cycle might enhance the positive climate-C cycle feedbacks (Reichstein *et al.*, 2013); however, increases in EE are predicted to cause significant losses of natural forests due to lagged forest migration (Malcolm *et al.*, 2002), increase in wildfires (Westerling *et al.*, 2006), increases in insect infestations and pathogen-related diseases (Kirilenko and Sedjo, 2007), and increase in forests'

production due to elevated $CO_2$ ($eCO_2$) "fertilization effect" (Idso and Kimball, 2001). Nonetheless, the mechanisms and the impacts of EE on the global C cycle are still not well understood due to inherently scarce data availability and the associated high uncertainties in the climate models (Seneviratne *et al.*, 2012).

Soil respiration ($R_S$), one of the least-studied studied elements of the terrestrial C cycle, is observed to be enhanced by $eCO_2$, high soil temperature, and after precipitation events (Birch effect), while is halted by low soil moisture (Orchard and Cook, 1983; Davidson *et al.*, 2000; Pregitzer *et al.*, 2008; Drake *et al.*, 2018). Nonetheless, the magnitude and tenacity of the $eCO_2$ and environmental variables' effects are highly dependable on the soil nutrient availability (Zak *et al.*, 2000, 2003; Finzi *et al.*, 2002; Hovenden *et al.*, 2008; Lagomarsino *et al.*, 2008). This is of high significance, especially for old-growth forests that might thoroughly have explored and exploited their belowground resources, limiting the possibility of increased C allocation belowground due to an increase in access to limiting nutrients (Körner, 2006). Moreover, nutrient cycles in mature forests are more coupled than young forests (Zaehle *et al.*, 2014), and mature trees have exhibited less flexibility in nutrient use (Körner *et al.*, 2005). Thus, understanding the nutrient mechanisms in mature forest ecosystems' capacity to sequester additional C is of great global concern and requires further investigation.

A series of extreme events happened at BIFoR FACE during the second year of $eCO_2$ enrichment; Beast from the East with low temperatures and snowfall until late March, a winter moth outbreak in mid-May, followed a six-week heatwave spell that lasted until early August. This is the first time that such a combination of extreme events occurred within a large-scale climate change experiment, thus provided the unique opportunity to study the combined impact of these extreme events *in situ* under $eCO_2$.

We measured $R_s$ at hourly intervals continuously across the second year of $CO_2$ enrichment at BIFoR FACE. We collected approximately 40k high-frequency automated measurements, offering precise inspection of the $R_s$ and the relationships with the environmental drivers and the potential interactions with $eCO_2$. Furthermore, we assessed the availability of ammonium ($NH_4^+$-N), nitrate ($NO_3^-$-N), and phosphate ($PO_4^{3-}$-P) at monthly intervals over the same period. We also performed three soil sampling campaigns assessing the microbial biomass throughout the second year of $CO_2$ enrichment.

## 4.3 Methodology

### 4.3.1 Site and extreme events description

A detailed description of the experimental site, Birmingham's Institute of Forest Research Free-Air Carbon Dioxide Enrichment (BIFoR FACE) facility, environmental drivers (VWC, $T_s$ and P), $R_s$, and soil biota available inorganic N and P can be found in Chapter 2 and 3 of the thesis. This Chapter focuses on data collected during the Dormant period (28th October 2017 – 25th March 2018) and Year 2 of $eCO_2$ enrichment (26th March 2018 – 31st October 2018), unless stated otherwise.

Briefly, BIFoR FACE is a temperate deciduous old-growth (>160 years) oak woodland located within Mill Haft at Staffordshire, England. The soil is brown earth luvisol with a sandy-clay texture and mean soil pH of 4.5 at the top 10 cm. Mean soil total N and P in the top 10 cm are 0.28% and 18 mg P $kg^{-1}$, respectively, indicating that BIFoR is a low nutrient woodland, and total organic matter content is 3.67%.

A detailed FACE facility description can be found in Hart *et al.* (2019). Briefly, the BIFoR FACE facility comprises six 30 m diameter infrastructure arrays (elevated $CO_2$

(*eCO₂* hereafter), n = 3; controls (*Ambient*, hereafter), n = 3) and three non-infrastructure arrays. Year 1 of $eCO_2$ enrichment began on 3rd April 2017 until 27th October 2017, while Year 2 began on the 26th March 2018 until 31st October 2018. $eCO_2$ enrichment took place from budburst to leaf fall approximately, and only during light hours, depending on the solar angle. During the first year of $eCO_2$ enrichment, $eCO_2$ arrays were within the 10% of target for 81.6% of the scheduled operation time and 20% of the target within 96.7% of scheduled operation time (Hart *et al.*, 2019). Contamination of the ambient arrays by $CO_2$ from the $eCO_2$ arrays was rare and short-lived, with ambient arrays being 10% of the control setpoint 98.8% of the time.

Figure 4-1 shows the schematic diagram of the study period timeline of Chapters 3 and 4 and the extreme events during Year 2 of $eCO_2$ enrichment at BIFoR FACE. The Pre-treatment period reflects the period before $eCO_2$ enrichment (19th October 2016 - 2nd April 2017), and Year 1 reflects the period when $eCO_2$ treatment was switched on (3rd April – 27th October 2017). Dormant reflects the period in-between Year 1 and Year 2 of $eCO_2$ enrichment (no $eCO_2$ enrichment during that period; 28th October 2017 – 25th March 2018). Finally, Year 2 reflects the second year of $eCO_2$ enrichment at BIFoR FACE (26th March 2018 – 31st October 2018).

Environmentally, 2018 was a unique year. The conditions varied greatly throughout the year, with the beginning of it having 0.2°C below the last 30-year average (*Met Office*, 2018). Three cold waves were experienced in a short period (late February until late March 2018); the Anticyclone Hartmut (known as the "Beast from the East"), Storm Emma followed by the mini Beast from the East. All three events were characterised by unusually low temperatures, strong winds, and heavy snowfall (*Beast from the East - Met Office*, 2018). The cold waves were displaced by warmer end of April and May 2018, with temperatures being 1° and 1.7° C above average (*Weather*

*review - Spring 2018* | *theWeather Club*, 2018). Late May/first week of June, BIFoR FACE experienced a winter moth (*Operopthera brumata*) outbreak causing a severe defoliation event. Lastly, the summer of 2018 was the warmest English summer alongside 1976, 2003, and 2006 (*2018 - A Record Breaking Year* | *theWeather Club*, 2018). From late June until early August 2018, UK experienced a six-week spell with an average temperature of 17.1 °C.

Figure 4-2 shows the air temperature, rainfall, snowfall, and sun hours from the closest to BIFoR FACE weather station (3.2 miles from BIFoR FACE, Gnosall, UK) since 2009 (*Gnosall, Staffordshire, United Kingdom Weather Averages* | *Monthly Average High and Low Temperature* | *Average Precipitation and Rainfall days* | *World Weather Online*, no date). The red box highlights the year 2018 for all the presented parameters. 2018 has the highest average temperature recorded (20 °C) since 2014. The driest month in 2018 was June, with monthly rainfall of 35.07 mm, whereas in all the previous years, the driest conditions were not during the summer season. Moreover, during 2018, the greatest amount of snow recorded since 2011, exceeding 15 cm coverage and the sun hours, remained higher than 200 from May until October 2018.


***Figure 4- 1:*** *Schematic diagram of the study period timeline (October 2016 – October 2018) and important extreme events.*

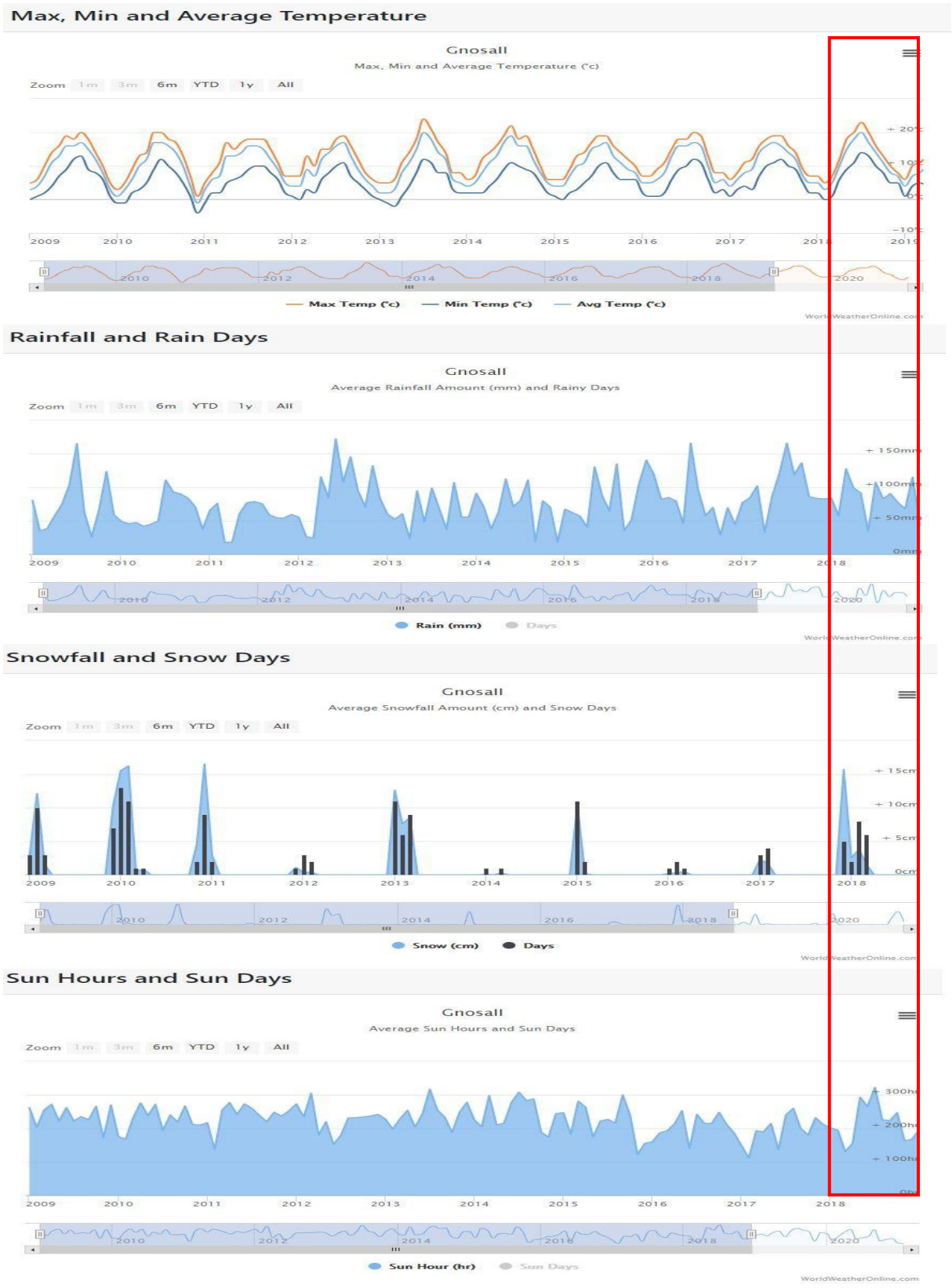


***Figure 4- 2:*** *Weather parameters (ait temperature, rainfall, snowfall and snow days, and sun hours) from a weather station 3.2 miles from BIFoR FACE from 2009 until 2018 (Gnosall, Staffordshire, United Kingdom Weather Averages | Monthly Average High and Low Temperature | Average Precipitation and Rainfall days | World Weather Online*, no date).

Figure 4-3 shows the canopy greenness index (GCC) from the PhenoCam installed at BIFoR FACE from October 2016 (Pre-treatment) until the end of Year 2 (October 2018). Daily GCC during Year 1 peaked at 0.46; however, during Year 2 reached 0.44, and the decrease was steeper than the one observed the year before.

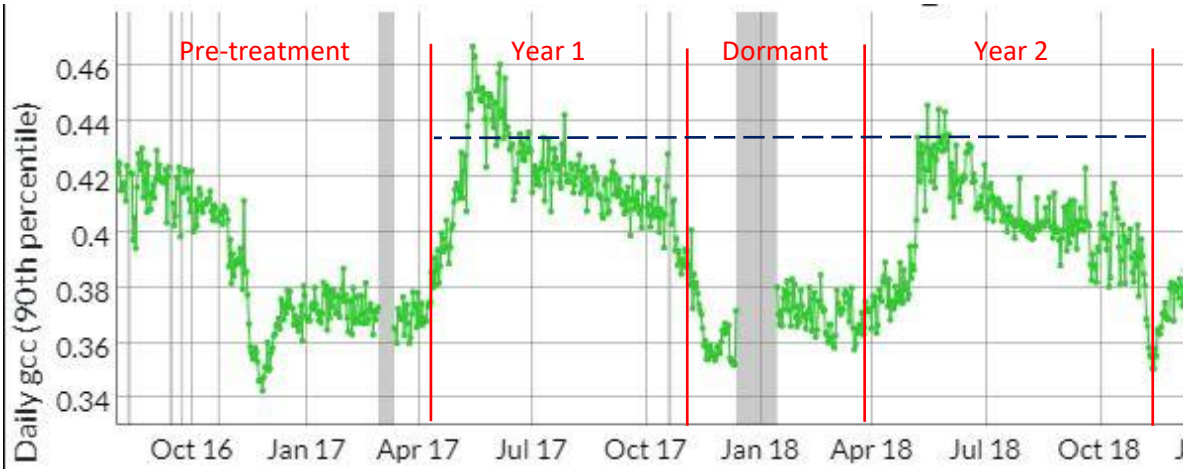


***Figure 4- 3:*** *Canopy "greenness index" at BIFoR FACE measured from PhenoCam showing the 90$^{th}$ percentile of the daily green chromatic coordinate (GCC) of the canopy phenology nine months before the $eCO_2$ switch-on until the end of the second year of $eCO_2$ enrichment. Data inside the red lines show the first and second years of the $eCO_2$ enrichment. The dashed line indicated the difference in the GCC between Year 1 and Year 2 of $eCO_2$ enrichment due to the extreme events. The grey lines indicate gaps in the dataset due to technical failures of the PhenoCam, while the red lines enclose the period of $eCO_2$ enrichment (https://phenocam.sr.unh.edu/webcam/sites/millhaft/)*

## 4.3.2 Measurements

### 4.3.2.1 $R_s$

Three surface PVC (Drainage Superstore, CMO Ltd, Plymouth, UK) collars for monitoring the $R_s$ were established within each of the six infrastructure arrays (n = 3, $eCO_2$ arrays, and n = 3, ambient arrays). The 20-cm-diameter collar was inserted 5 cm into the soil and permitted the measurement of the total $R_s$. The collars were installed in spring 2016 and left for establishment through the growing season; the soil collars were permanently installed to reduce soil disturbance by repeated

measurements and ensure that the same soil was measured over time (King *et al.*, 2004).

Automated measurements of $R_s$ were taken at 1-hour intervals at all three surface collars in an $eCO_2$ array and its paired ambient array simultaneously, using 20-cm-diameter long-term chambers interfaced with a multiplexer and an infra-red gas analyser (IRGA) (Li-8100-104 long-term chambers, Li-8150 multiplexer, and Li-8100A IRGA; LI-COR). The observation length was 2 min with 20 s dead-band, a 15 s pre-purge, and a 45 s post purge, giving a total measurement cycle of 3 min 20 s per collar. The measurements began on 19th October 2016 (5 months before the beginning of $eCO_2$ enrichment), and we report data until 31st October 2018 (end of Year 2 of $eCO_2$ enrichment). The LI-COR systems were moved every two weeks between replicate paired $eCO_2$ and ambient arrays, returning to the same array pair on a 4-week rotation.

All data went through quality checks before analysis using SoilFluxPro software, version 4.0.1 (LI-COR, Nebraska, USA). Data points with negative linear fluxes or data points with a linear flux coefficient of variation higher than 3.5 were removed from the dataset, as these were indicative of leaks in the chamber. Data points with a linear flux coefficient of variation between 1.5 and 3.5 were manually checked for assessing the fit quality. Of a total of 43,776 data points during the second year of operation, 27,217 data points were used for analysis. There was a moderate data loss (38%), mostly due to equipment failures (water condensation in the electric plates) and, to a less extent, due to poor-quality fits. Daily and monthly averages of these data are reported in this study in μmol $CO_2$ $m^{-2}$ $s^{-1}$.

#### 4.3.2.2 Environmental drivers

Soil volumetric water content (VWC) and soil temperature ($T_s$) were measured with permanently installed shallow CS655 probes inserted in the top 12 cm soil (Campbell Scientific, Logan, UT, USA). VWC probes were sited one meter from each of the three experimental $R_s$ collars in each array. The VWC data were recorded in 15-min intervals by a data logger in each plot (CR1000, Campbell Scientific) and reported daily.

The temperature sensitivity ($Q_{10}$) of $R_s$ was calculated using the following equations

$$lnR_s = kT + C$$

$$Q_{10} = e^{10k}$$

where k is the regression slope of ln-transformed respiration against measured temperature, and *C* is an empirical constant (Kutsch, Bahn and Heinemeyer, 2010).

#### 4.3.2.3 Bioavailable inorganic N and P

Ion-exchange resin membranes (Membranes International Inc., New Jersey, USA) were used to measure the available inorganic N, as ammonium ($NH_4^+$-N) and nitrate ($NO_3^-$-N), and P, as phosphate ($PO_4^{3-}$-P). Three anion- (AMI 7001; 2 cm x 12 cm) and three cation-exchange (CMI 7000; 2 cm x 12 cm) resin membranes were inserted in the soil at eight locations within each array; three sets of each were located next to the $R_s$ collars, and the other five sets were randomly located within each the array. Resin membranes were deployed in situ for approximately one month before retrieval.

Upon retrieval, resin membranes were washed thoroughly with deionised water to remove any soil particles and then extracted for $PO_4^{3-}$, $NH_4^+$ and $NO_3^-$ (modified from Rayment and Lyons (2011) and Bowatte *et al.* (2008), respectively. The three resin membranes from each location were bulked together as one sample, extracted with 0.5 M HCl, shaken at 180 rpm for two hours, and then the resin membranes were removed from the solution and discarded.

Concentrations of $NH_4^+$ and $NO_3^-$ were analysed by a continuous flow analyser (San++ Continuous Flow Analyser, Skalar, Breda, The Netherlands), and concentrations of $PO_4^{3-}$ were analysed by ultraviolet-visible spectroscopy (Jenway) using the molybdenum blue method (Worsfold *et al.*, 2005). The data presented in this study are reported as monthly averages in μg $NH_4^+$-N $cm^{-2}$ $d^{-1}$, μg $NO_3^-$-N $cm^{-2}$ $d^{-1}$, and μg $PO_4^{3-}$-P $cm^{-2}$ $d^{-1}$, respectively, and the ratios of these.

#### 4.3.2.4 Microbial biomass and inorganic N

Three soil sampling campaigns took place during Year 2 of $eCO_2$ enrichment, one in spring (6th March 2018), one in summer (9th July 2018), and one in autumn (26th November 2018). For each campaign, one soil core (30 cm long x 55 mm ø) was collected from three soil plots in each of the ambient and $eCO_2$ arrays (18 cores in total). Each core was subsequently separated into O, A, and B horizons (54 samples).

From each sample, six subsamples were taken; one for determining soil moisture gravimetrically, four for determining microbial biomass C, N, and P, and inorganic $NH_4^+$-N and $NO_3^-$-N, and one for DNA analysis (data not shown). For determining the gravimetric volume content, ~5 g of soil were weighted in a tin cup and kept in an oven

for 48 h at 105 °C. After 48 h, the samples were reweighted, and the gravimetric volume content was calculated using the following equation:

$$\text{Gravimetric volume content} = \frac{(wt\ of\ wet\ soil + tare) - (wt\ of\ dry\ soil + tare)}{(wt\ of\ dry\ soil + tare) - tare}$$

For assessing microbial biomass C, N, and P and inorganic $NH_4^+$-N and $NO_3^-$-N, four subsamples were taken and separated into chloroform fumigated and non-fumigated. For both fumigated and non-fumigated samples, the weights differed by horizon (2 g for O horizon and 5 g for A and B horizons). The chloroform fumigated samples were transferred into glass vials and were placed in a vacuum desiccator with 20 mL of ethanol-free chloroform. The desiccator was evacuated until the chloroform was boiling vigorously, and this process was repeated three times. The desiccator was left in the dark for 48 hours on the fourth evacuation, with the valve closed to prevent chloroform from breaking down. In the meantime, the non-fumigated samples were incubating in the dark next to the vacuum desiccator.

After 48 hours, one set of chloroform fumigated and non-fumigated samples were extracted with 10 mL of 0.5 M $K_2SO_4$, syringe filtered, and the filtered extracted was analysed for total organic carbon (TOC) and total organic nitrogen (TON) using Analytik Jena Multi N/C series (Analytik Jena, Jena, Germany). Only the non-fumigated samples were analysed for inorganic $NO_3^-$-N and $NH_4^+$ using the same method described previously in the *Biota available (bioavailable) inorganic N and P* section.

The other chloroform fumigated and non-fumigated set of samples was used to analyse $PO_4^{3-}$-P. After 48 hours of incubating, an anion-exchange resin membrane (AMI 7001, Membranes International Inc., New Jersey, USA; 2 cm x 4 cm) was added to the sample with 15 mL deionised water and were shaken for 2 hours at 200 rpm. After 2 hours, the samples were kept for further total P analysis (data not shown), while the anion-exchange resin membranes were washed and transferred in new sample tubes. Anion-exchange resin membranes were extracted with 0.5 M HCl and shaken for 24 hours at 180 rpm. After 24 hours, the anion-exchange resin membranes were discarded, while the solution was used to determine the $PO_4^{3-}$-P using the same method as described previously in the *Biota available (bioavailable) inorganic N and P* section.

## 4.3.3 Statistical analysis

All analyses were carried out using R 3.6.2 and Rstudio 1.2.5033 (RStudio Team, 2019). The mixed-effects models were performed using R package lme4 (Bates *et al.*, 2015), and the coefficient of determination was calculated using the R package MuMIn (Barton, 2014). For all analyses, effects were considered to be strongly significant if the model-reported p-value < 0.05 and marginally significant if p-value < 0.1.

### 4.3.3.1 Environmental drivers and $R_s$

Treatment effects were derived directly by comparing the daily means of $R_s$ for $eCO_2$ and ambient arrays using a one-way ANOVA. The dormant period and Year 2 were assessed separately to establish potential significant differences during switch-on and -off eCO2 enrichment and significant treatment effects. Interactions between $eCO_2$

and environmental drivers (VWC and $T_s$) were evaluated using mixed-effects models. In all mixed-effects models performed, 'array,' 'location within the array,' and 'number of days since the beginning of the experiment' to account for the correlations between repeated measurements within each collar/array were added as random effects. All mixed-effects models performed in this Chapter daily averages of $R_s$ (per collar), VWC, and $T_s$ (per sensor, respectively) were used.

The relationships between $R_s$ and VWC as well as $R_s$ and $T_s$ were linear during the Dormant period. The AIC tool was used to determine which of the models mentioned above was most likely the best model for this dataset. The two models with the lowest AIC score were compared using the ANOVA function to compute the Chi-squares between the two models for investigating the model fit. Finally, the coefficient of determination ($R^2$) was calculated to assess the variance captured by the model.

Similarly, the same principles as described above were used for investigating the $eCO_2$ effects during Year 2 of $eCO_2$ enrichment at BIFoR FACE. $eCO_2$, its interaction with VWC and $T_s$, and the complex interaction between all three variables were used as fixed effects. However, during Year 2, only Rs and Ts' relationship was linear, whereas $R_s$ and VWC was quadratic. The AIC tool was utilised again to choose the most likely best model amongst the ones mentioned above, while the ANOVA function was used for concluding the best-fitted model.

#### 4.3.3.2 Estimate of annual $R_s$

Due to the experimental setup (measuring one ambient array and one $eCO_2$ array for two weeks) and given the seasonal and temporal variability of $R_s$, a modelled approach was used to calculate the seasonal $R_s$, utilising the best-fitted models described in

section *4.3.3.1 Environmental drivers and $R_s$*. Daily averages of the $R_s$ data were estimated using the best-fitted models for Dormant and Year 2, predicting the $R_s$ of the rest of the arrays at all periods for the whole study period. VWC and $T_s$ from each array aggregated in daily averages were used as predictors. Subsequently, daily averages of observed and predicted $R_s$ values were merged, producing a complete $R_s$ dataset.

#### 4.3.3.3 Soil bioavailable nutrients

For nutrient analysis, the replication unit was the FACE array (n=3 for each ambient and $eCO_2$), and all data were aggregated in monthly timestamps. The effect of $eCO_2$ only on bioavailable nutrient concentrations was assessed using linear-mixed-effects models, for which 'array' and 'number of days since the beginning of the measurements' were random factors. Data collected before the $eCO_2$ enrichment were analysed separately to assess potential hereditary $eCO_2$ differences. Subsequently, linear-mixed-effects models were undertaken with $CO_2$ and two covariates – $T_s$ and VWC – and their interactions ($eCO_2$*$T_s$, $eCO_2$*VWC, and $eCO_2$*$T_s$ *VWC) as fixed effects to evaluate the role of soil conditions in responses to elevated $CO_2$. In this mixed-effects framework, 'array' and 'number of days since the beginning of the measurements' were used as random factors.

#### 4.3.3.4 Microbial biomass and inorganic N

Similarly, for the soil intensives' analysis, the replication unit was the FACE array (n=3 for each of ambient and $eCO_2$). We utilised the linear-mixed-effects models again to assess the $eCO_2$ effect on microbial biomass and inorganic N, for which 'array' and

'campaign' were random factors, while 'soil horizon', 'season' and '$eCO_2$' were fixed factors.

## 4.4 Results

### 4.4.1 $R_s$ response to environmental drivers and $eCO_2$

$R_s$ exhibited high variability throughout the study period, following seasonal patterns related to VWC, $T_s$, and precipitation patterns (Fig. 4-4). A detailed description of the Pre-treatment period and Year 1 can be found in Chapter 3. The Dormant period started on 28th October 2017 and lasted until early spring (25th March 2018). During that period, no $eCO_2$ was introduced in the woodland. The dashed lines on Fig. 4-4 enclose the $eCO_2$ enrichment period (Year 1 3rd April – 27th October 2017; Year 2 26th March – 31st October 2018). The dotted black rectangle encloses the defoliation event, while the dotted orange rectangle encloses the heatwave event.

$R_s$ increased at the beginning of the growing season (concurring with $eCO_2$ enrichment) in both ambient and $eCO_2$ treatments. $T_s$ increased, and VWC decreased, followed by a steep decrease as the VWC decreased from approximately 20 to 10% in mid-May (Fig. 4-4a-c). $R_s$ increased again as VWC and $T_s$ increased during the defoliation event, only to decrease further during the summer when $T_s$ was over 15 °C, VWC dropped below 10%, and there were no Pr events. $R_s$ increased again in the autumn as the VWC started to rise again, and Pr events were more frequent.

Both daily and monthly averages indicated that Rs was frequently higher in the $eCO_2$ arrays. The greatest differences appeared to be during May and August 2018 (May 1.56 µmol $CO_2$ $m^{-2}$ $s^{-1}$ in ambient vs. 3.33 µmol $CO_2$ $m^{-2}$ $s^{-1}$ in $eCO_2$; August 2.14 µmol $CO_2$ $m^{-2}$ $s^{-1}$ in ambient vs. 3.60 µmol $CO_2$ $m^{-2}$ $s^{-1}$ in $eCO_2$). Precipitation was observed

to lead to a pulse effect on $R_s$ commensurate with an increase in VWC. $T_s$ had a strong and clear seasonal pattern, whereas VWC had a steep decrease lower than 10% for approximately five months (July – October 2018). Pr was sporadic and highly variable, and no Pr events were recorded for around four weeks (Fig. 4-4b, c).

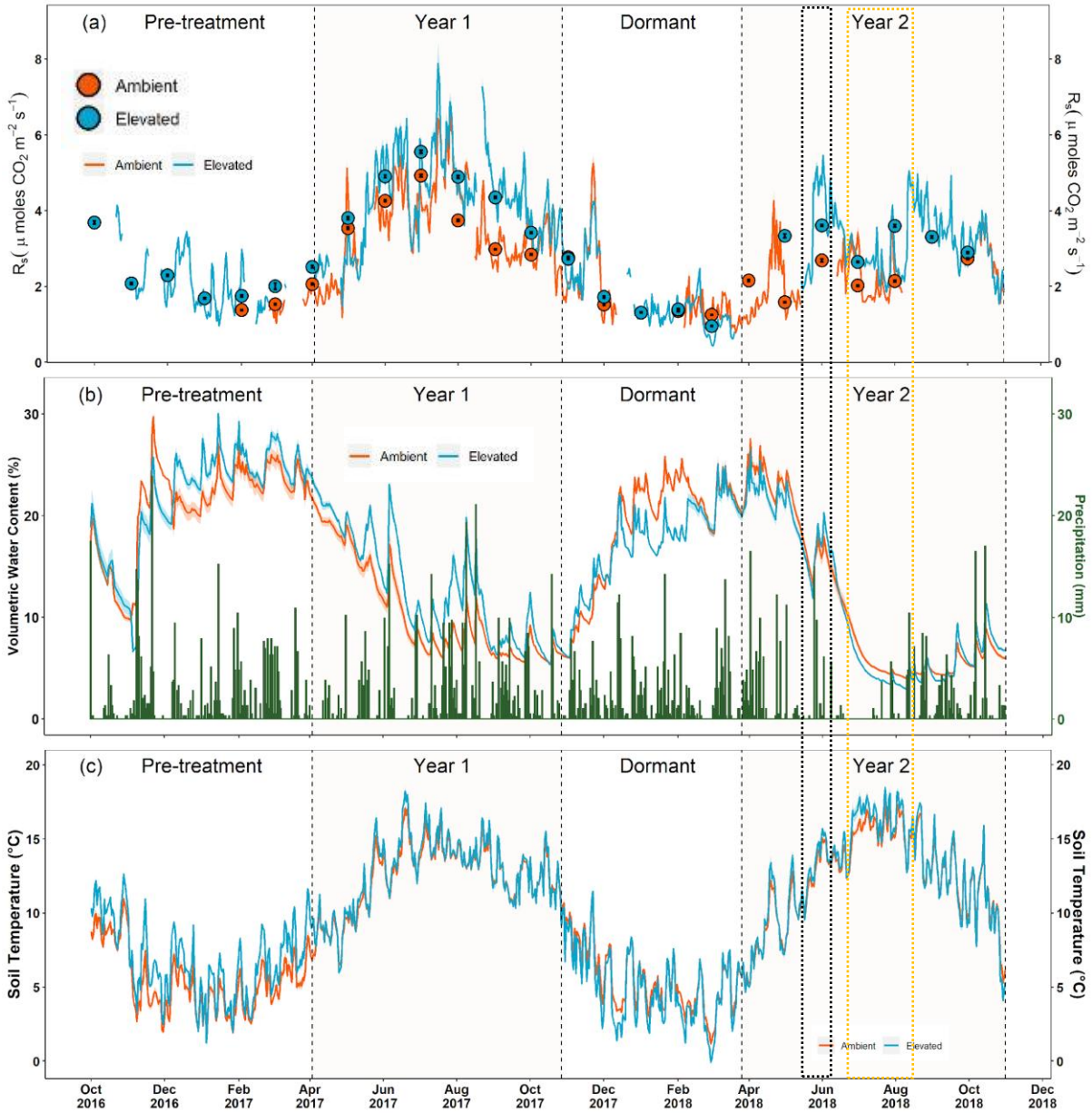


**Figure 4- 4:** *Seasonal course of $R_s$ (a), VWC and precipitation (b), and $T_s$ (c) at BIFoR FACE during the first two years of $eCO_2$ enrichment (October 2016 – October 2018). The blue coloured lines/solid points denote the $eCO_2$ arrays, while the orange lines/solid points denote the ambient arrays. The lighter coloured ribbons around the lines and the error bars indicate the se for $R_s$, VWC, and $T_s$. All data describe daily averages, except the solid points in (a), which reflect the $R_s$ monthly averages. The vertical dashed lines enclose the period when the $eCO_2$ enrichment was switched on (Year 1, 3rd April – 27th October 2017 and Year 2, 26th March – 31st October 2018), whereas the periods in between the dashed segments reflect*

*the Pre-treatment period (19th October 2016 – 2nd April 2017) and the Dormant period (28th October 2017 – 25th March 2018).*

Table 4-1 shows the average (mean ± standard deviation), minimum and maximum VWC, $T_s$, and $R_s$ during Dormant and Year 2 (2018), and the $Q_{10}$ values both ambient and $eCO_2$ arrays. Mean VWC, $T_s$, and $R_s$ were similar between ambient and $eCO_2$ arrays, with $T_s$ and $R_s$ having a greater range and variability in $eCO_2$ arrays, whereas VWC had a greater range in ambient arrays. $Q_{10}$ was higher in ambient arrays during Dormant (3.54 in ambient vs. 4.28 in $eCO_2$ arrays). During Year 2, $eCO_2$ arrays were drier and warmer, on average, than ambient arrays, while $Q_{10}$ was higher in ambient arrays (1.40 in ambient vs. 1.23 in $eCO_2$ arrays).

Simple mixed-effects models were used to investigate significant differences in daily VWC, $T_s$, $R_s$ between ambient and $eCO_2$ arrays for both Dormant and Year 2. VWC and $R_s$ were statistically similar between ambient and $eCO_2$ arrays during the Dormant period (p-value 0.71 and 0.84, respectively; Table 4-1), while $T_s$ was marginally significant (p-value 0.05). During Year 2 $R_s$, there was a statistical difference between ambient and $eCO_2$ arrays (p-value 0.02), while VWC and $T_s$ were statistically similar (p-value 0.9 and 0.5, respectively).

***Table 4- 1:*** *Mean, minimum and maximum VWC, $T_s$, and $R_s$ for Dormant and Year 2 in ambient and $eCO_2$ arrays based on daily averages (mean ± sd), as well as $Q_{10}$ values and p-values from simple mixed-effects models. Significance values are based on the mixed-effects model approach with $eCO_2$ as the main effect and array, location within an array, and the number of days since the beginning of the measurement as random effects. p-value <0.1 is shown in bold.*

| Variable | Mean | | Minimum | | Maximum | | p-value |
|---|---|---|---|---|---|---|---|
| | Ambient | $eCO_2$ | Ambient | $eCO_2$ | Ambient | $eCO_2$ | |
| *Dormant* | | | | | | | |
| VWC | 16.66±6.00 | 17.13±4.62 | 6.00 | 6.05 | 25.10 | 25.04 | 0.71 |
| $T_s$ | 5.82±2.44 | 5.13±2.47 | 1.16 | -0.07 | 11.08 | 11.46 | **0.05** |
| $R_s$ | 1.8±0.92 | 1.68±0.83 | 0.78 | 0.43 | 5.24 | 4.25 | 0.84 |
| $Q_{10}$ | 3.54 | 4.28 | | | | | |
| *Year 2* | | | | | | | |
| VWC | 13.12±8.3 | 7.66±4.59 | 4.13 | 3.00 | 27.57 | 20.29 | 0.9 |
| $T_s$ | 11.86±3.68 | 13.62±2.94 | 5.05 | 4.09 | 17.48 | 18.47 | 0.5 |
| $R_s$ | 2.20±0.74 | 3.20±0.88 | 1.01 | 1.54 | 4.26 | 5.46 | **0.02** |
| $Q_{10}$ | 1.40 | 1.23 | | | | | |

Fig 4-5 shows a preliminary analysis of $R_s$ to VWC and $T_s$ individually. The data presented in this graph are only during the second $eCO_2$ enrichment period (*Year 2*; 26th March – 31st October 2018) to assess both ambient and $eCO_2$ conditions and exclude the Dormant period (low $T_s$ and high VWC) in an attempt to isolate each drivers effect.

A positive effect of VWC on $R_s$ rates (Fig. 4-5a) was identified under low VWC levels (<10%) under ambient conditions, followed by a plateau response until ~ 14%. As the VWC levels increased further than those levels, the effect of VWC on $R_s$ was negative. Although it appears as the VWC in higher levels had a negative impact on $R_s$, the VWC levels were not that high as to prohibit $R_s$. Thus this response suggests an

interaction with $T_s$. Under $eCO_2$ conditions, the VWC effect on $R_s$ was positive across the whole range of VWC.

The effect of $T_s$ on $R_s$, under ambient conditions, was positive until ~ 13 °C, followed by a negative effect as the $T_s$ increased further (Fig. 4-5b). Similarly, under $eCO_2$ conditions, $T_s$ had a positive effect on $R_s$ rates until ~ 15 °C (2 °C more than under ambient conditions), followed by a steeper than under ambient conditions negative effect as $T_s$ increased further.

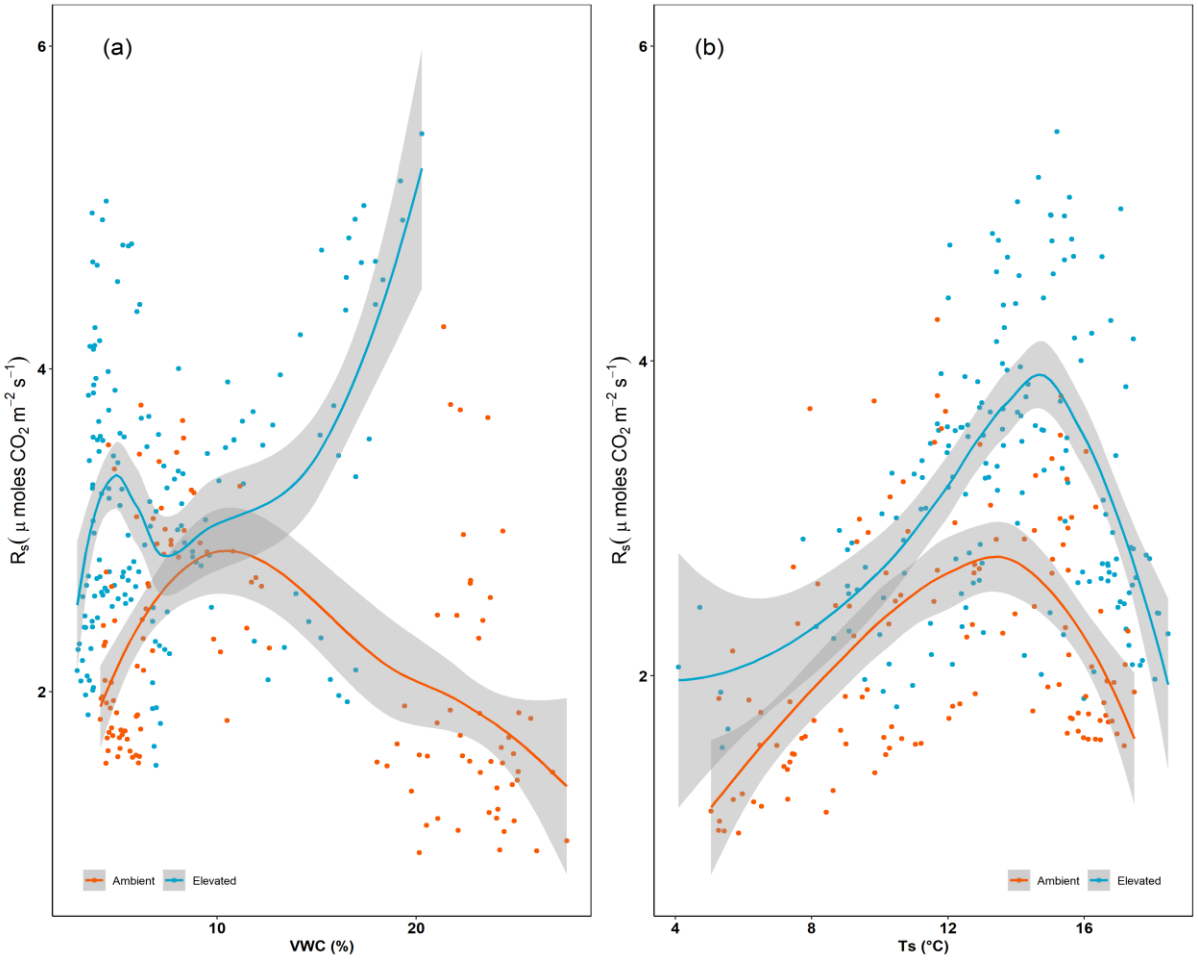


***Figure 4- 5:*** *$R_s$ response (LOESS) on VWC (a) and $T_s$ (b) in both $eCO_2$ and ambient conditions during Year 2. The blue solid points represent the $eCO_2$ arrays, while the orange solid points represent the ambient arrays.*

The synergic effects of $eCO_2$ treatment on $R_s$ with the environmental drivers (VWC and Ts) were also explored using more complex models that incorporated interaction effects. The dual impact of $eCO_2$ and each environmental driver and their triadic interaction can be found in Table 4-2. During Dormant, $eCO_2$ had no statistically significant effect on $R_s$ (p-value 0.85) but all interactions ($eCO_2$*VWC, $eCO_2$*$T_s$ and $eCO_2$*VWC*$T_s$) were statistically significant (p-value 0.07, 0.0001 and 0.005, respectively). The two models with the lowest AIC were the $eCO_2$*$T_s$ and $eCO_2$*VWC*$T_s$; however, the ANOVA function showed that the best-fitted model was the latter explaining 73% of the observed variability on $R_s$. The model was validated for homoscedasticity, normality, the linearity of residuals, and the random effects' linearity (see section 4.8 Supplementary Material, Figure S4-1).

During Year 2, $eCO_2$ had a statistically significant effect on $R_s$ (p-value 0.02), while only the $eCO_2$*VWC and $eCO_2$*VWC*$T_s$ interactions were statistically significant (p-value <0.001 for both). These two interaction models were also the ones with the lowest AIC and could explain 49 and 60% of $R_s$' variability. The ANOVA function was used to determine which of the two models was the best fitted; the latter model was the best (p-value < 0.001). Finally, the model was validated for homoscedasticity, normality, the linearity of residuals, and the random effects' linearity (see section 4.8 Supplementary Material, Figure S4-2).

***Table 4- 2:*** *Analysis of $R_s$ and its drivers' variance during Dormant and Year 2 at BIFoR FACE. The degrees of freedom for each explanatory variable is 1, p-value < 0.1 is shown in bold.*

| Predictors | $DF_{den}$ | F-value | p-value | $R_c^2$ | AIC |
|---|---|---|---|---|---|
| *Dormant* | | | | | |
| $eCO_2$ | 4.00 | 0.04 | 0.85 | 0.42 | 1607.58 |
| $eCO_2$*VWC | 579.87 | 3.16 | **0.07** | 0.64 | 1281.34 |
| $eCO_2$*$T_s$ | 578.45 | 14.88 | **0.0001** | 0.70 | 1025.81 |
| $eCO_2$*VWC*$T_s$ | 584.81 | 7.88 | **0.005** | 0.73 | 1047.75 |
| *Year 2* | | | | | |
| $eCO_2$ | 4.00 | 14.73 | **0.02** | 0.45 | 2289.99 |
| $eCO_2$*VWC | 855.28 | 73.04 | **<0.001** | 0.49 | 2288.20 |
| $eCO_2$*$T_s$ | 847.8 | 1.01 | 0.31 | 0.49 | 2293.32 |
| $eCO_2$*VWC*$T_s$ | 848.54 | 61.03 | **<0.001** | 0.60 | 2163.37 |

*Where $DF_{den}$: denominator's degrees of freedom, F-value: F-test determination, $R_c^2$: coefficient of determination, and AIC: Akaike Information tool*

Utilising the models' from above, we were able to estimate the $R_s$ by imputing data for the arrays that were not measured each fortnight or periods where no data existed and create a "complete" dataset. Thus, at all times, we have n=3 with two arrays being predicted values and the third one being the observed values for ambient and $eCO_2$ arrays, respectively.

### 4.4.2 Annual $R_s$ estimates

As mentioned in the section above, the best-fitted models were utilised to predict Rs' missing value$_s$ for the studied period. Figure 4-6a and b show the observed $R_s$ values against the predicted $R_s$ values in ambient and $eCO_2$ arrays during both Dormant and Year 2 of $eCO_2$ enrichment at BIFoR FACE. Note that in Fig. 4-6a during Year 2, the model cannot capture the temporal variability due to missing data. The observed values are daily averages of one array each fortnight, whereas the predicted values

are daily averages of three arrays for ambient and $eCO_2$ arrays, respectively. The objective of the model was to "fill the gaps" between $R_s$ measurements with defensible estimates. Observed and predicted $R_s$ were strongly correlated (Fig. 4-6c; $p<0.001$ for both ambient and $eCO_2$ arrays; $R = 0.76$ and 0.83, respectively); the equation's slope was 0.96 and 1 for ambient and $eCO_2$ arrays, respectively.

Rs' mean annual flux was estimated as the predicted and observed daily averages of $R_s$ data above (Fig. 4-6d). The mean annual $R_s$ at BIFoR FACE during Year 2 in ambient arrays was 713 ±15 g C $m^{-2}$ $y^{-1}$ (mean±se), while in $eCO_2$ arrays was approximately 36.5% higher (973 ±19 C $m^{-2}$ $y^{-1}$; mean±se).

### 4.4.3 Bioavailable nutrients

All three soil bioavailable nutrients ($NO_3^-$-N, $NH_4^+$-N, and $PO_4^{3-}$-P) exhibited strong seasonality (Fig. 4-7a, b, and c), demonstrating a similar seasonal pattern with $R_s$ and VWC (see Fig. 4-4). All three bioavailable nutrients had low availabilities at the beginning of the $eCO_2$ enrichment (April 2018; $NO_3^-$-N 5.6±0.4 in ambient vs. 5.5±0.6 µg $NO_3^-$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays; $NH_4^+$-N 2.0±0.3 in ambient vs. 3.9±0.7 µg $NH_4^+$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays; $PO_4^{3-}$-P 0.4±0.1 in ambient vs. 0.5±0.1 µg $PO_4^{3-}$-P $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays). All three nutrients availabilities increased until June, followed by a dramatic decrease in July 2018 which was more intense in $eCO_2$ arrays ($NO_3^-$-N 4.1±0.5 in ambient vs. 2.2±0.2 µg $NO_3^-$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays; $NH_4^+$-N 3.9±0.4 in ambient vs. 3.3±0.3 µg $NH_4^+$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays; $PO_4^{3-}$-P 0.33±0.05 in ambient vs. 0.24±0.03 µg $PO_4^{3-}$-P $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays). The decrease in ambient arrays was 76, 46, and 79% for $NO_3^-$-N, $NH_4^+$-N, and $PO_4^{3-}$-P, respectively, whereas in $eCO_2$ arrays was 80, 60, and 85% for $NO_3^-$-N, $NH_4^+$-N, and $PO_4^{3-}$-P, respectively. In August and September, all nutrient availabilities increased as VWC and Pr increased. $NO_3^-$-N availability increased 7 vs. 14 times in ambient and $eCO_2$ arrays, respectively, $NH_4^+$-

N increased 3 times in both ambient and $eCO_2$ arrays, while $PO_4^{3-}$-P increased 10 vs. 20 times in ambient and $eCO_2$ arrays, respectively. Accordingly, N:P ratios decreased as the nutrients' availability increased and vice versa (Fig. 4-7d).

All three nutrients availabilities followed similar patterns in ambient and $eCO_2$ arrays, and for the majority of Year 2 was challenging to identify clear separations between ambient and $eCO_2$ arrays. However, the greatest differences for $NO_3^-$-N availability were observed in June 2018 (17.5±1.6 in ambient vs. 11.4±0.9 µg $NO_3^-$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays, respectively), for $NH_4^+$-N in September 2018 ($NH_4^+$-N 14.5±2.5 in ambient vs. 12.6±2.1 µg $NH_4^+$-N $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays, respectively), and $PO_4^{3-}$-P was in August 2018 ($PO_4^{3-}$-P 3.1±0.4 in ambient vs. 5.0±0.6 µg $PO_4^{3-}$-P $cm^{-2}$ $d^{-1}$ in $eCO_2$ arrays).

Table 4-3 shows the averages during Dormant and Year 2 of all three soil bioavailable nutrients and N:P ratios in ambient and $eCO_2$ arrays. No statistically significant differences were observed in the availability of all three nutrients and N:P ratios during both Dormant and Year 2.

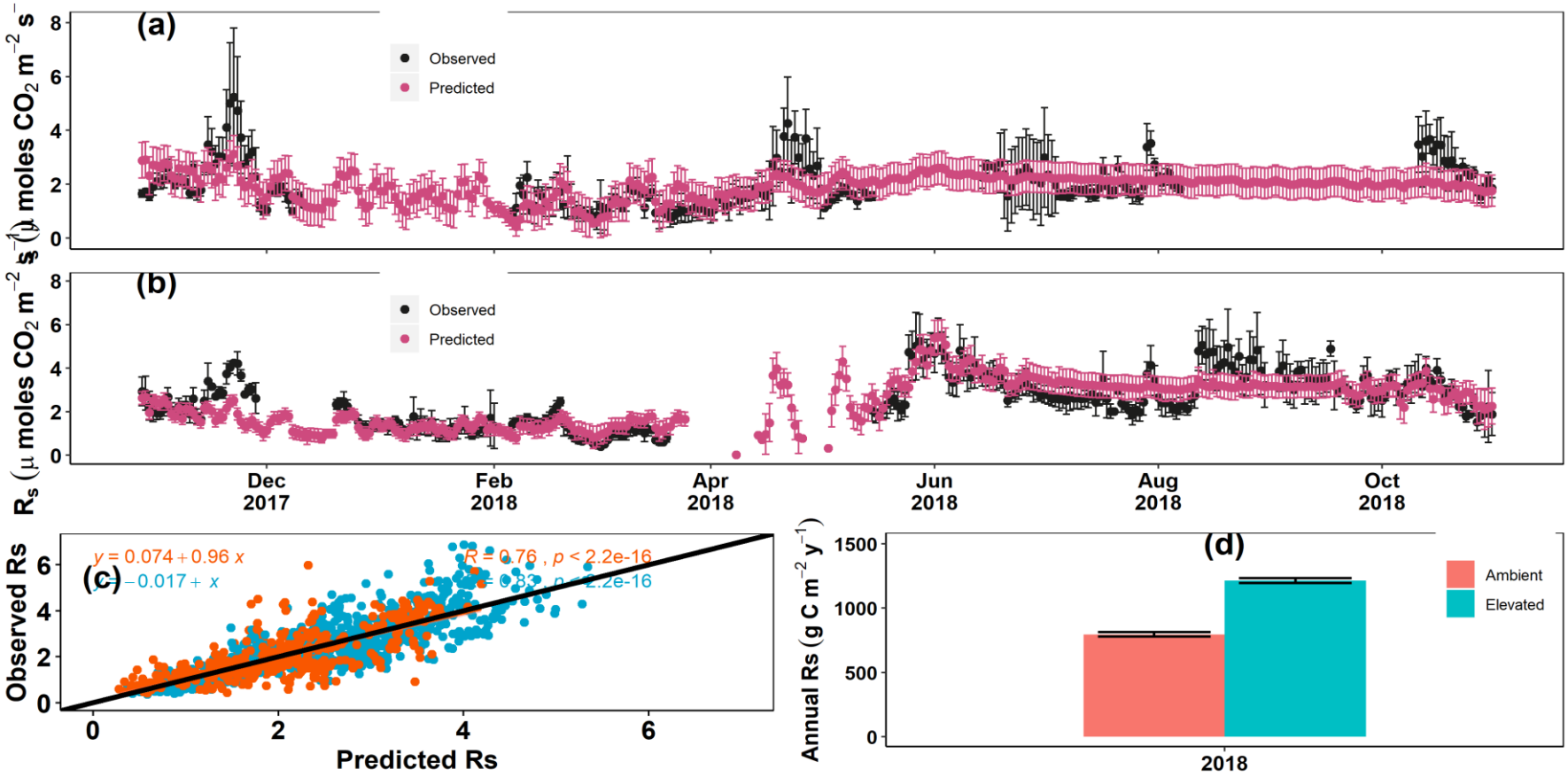


***Figure 4- 6:*** *a) Observed and predicted $R_s$ measurements under ambient conditions, b) Observed and predicted $R_s$ measurements under $eCO_2$ conditions. The black solid points denote the daily-average (mean ± sd) observed $R_s$ measurements, while the pink solid points denote the predicted $R_s$ values. The dashed lines enclose Year 1 of $eCO_2$ enrichment. c) Predicted and observed $R_s$ correlation for $eCO_2$ (blue line) and ambient (orange line) arrays. The blue solid points denote the $eCO_2$ arrays, while the orange solid points denote the ambient arrays. d) Mean annual $R_s$ in g C $m^{-2}$ $y^{-1}$ in ambient (orange) and $eCO_2$ (blue) arrays during the second year at BIFoR FACE. The error bars denote the standard error.*

***Table 4- 3:*** *Soil nutrients availability and N:P ratios during the Dormant and Year 2 at ambient and $eCO_2$ arrays (mean ± sd), and summary of mixed-effects models of the $eCO_2$ effect on nutrient availability. All nutrients units are per unit area of membrane in µg $cm^{-2}$ $d^{-1}$(refer to section 4.3.3.3 Bioavailable nutrients for method). p-value < 0.1 is shown in bold.*

| | | | | **$eCO_2$ effect** | |
|---|---|---|---|---|---|
| **Variable** | **Ambient** | **$eCO_2$** | **$DF_{den}$** | **F-value** | **p-value** |
| *Dormant* | | | | | |
| $NO_3^-$ - N | 12.4 ± 9.6 | 8.9 ± 7.7 | 20 | 1.47 | 0.24 |
| $NH_4^+$ - N | 2.4 ± 1.6 | 2.3 ± 1.3 | 3.93 | 0.05 | 0.83 |
| $PO_4^{3-}$ - P | 0.7 ± 0.8 | 0.6 ± 0.9 | 3.97 | 0.71 | 0.45 |
| log(N:P) ratio | 3.4 ± 0.9 | 3.2 ± 1.0 | 3.93 | 0.05 | 0.84 |
| *Year 2* | | | | | |
| $NO_3^-$ - N | 17.9 ± 13.0 | 15.3 ± 12.9 | 40 | 0.43 | 0.52 |
| $NH_4^+$ - N | 6.3 ± 4.5 | 6.4 ± 3.4 | 40 | 0.004 | 0.94 |
| $PO_4^{3-}$ - P | 1.9 ± 2.0 | 2.1 ± 2.1 | 40 | 0.05 | 0.82 |
| log(N:P) ratio | 2.9 ± 0.5 | 2.8 ± 0.6 | 4 | 0.24 | 0.65 |

*Where $DF_{den}$: denominator's degrees of freedom and F-value: F-test determination*

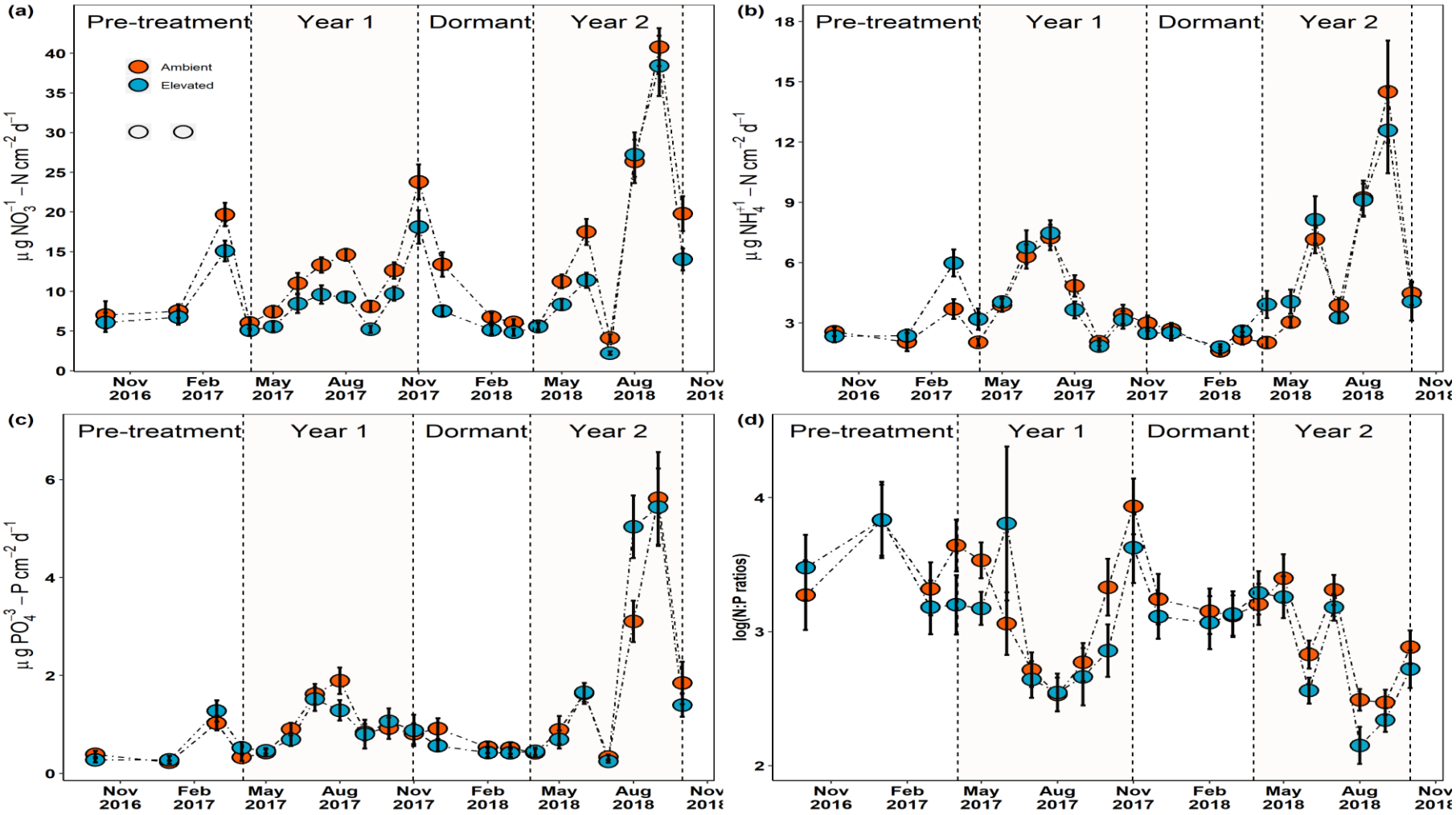


***Figure 4- 7:*** *Temporal changes in ion exchange resin membranes (a) $NO_3^-$-N, (b) $NH_4^+$-N and (c) $PO_4^{3-}$-P concentrations, (d) log(N:P ratios), (mean ± se, n = 3). The orange solid points reflect the nutrient availability in the ambient arrays, whereas the blue solid points reflect the nutrient availability in the $eCO_2$ arrays. The shaded area denotes eCO2 treatment (Year 1 and Year 2), whereas the white areas reflect the Pre-treatment and Dormant period (no $eCO_2$ enrichment)*

VWC and Pr had a statistically significant effect on all three bioavailable nutrients ($NO_3^-$-N, $NH_4^+$-N, and $PO_4^{3-}$-P) as well as the N:P ratio (Table 4-4). $eCO_2$, $T_s$, and the interactions of $eCO_2$ with the environmental variables ($eCO_2$*VWC, $eCO_2$*$T_s$, and $eCO_2$*Pr) had no statistically significant effect on neither nutrients availabilities nor on N:P ratios.

**Table 4- 4:** Summary of mixed-effects models of the $eCO_2$, environmental variables, and their interactions with $eCO_2$ on bioavailable soil nutrients. Fixed factors are $eCO_2$, VWC, $T_s$ and their interactions with $CO_2$. The degrees of freedom for each explanatory variable are 1. p-value<0.1 shown in bold.

| | | Predictors | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Response variables | | $eCO_2$ | VWC | $T_s$ | Pr | $eCO_2$*VWC | $eCO_2$*$T_s$ | $eCO_2$*Pr |
| $NO_3^-$-N | t | -0.66 | -2.19 | -1.49 | 2.54 | -0.05 | 0.66 | 0.30 |
| | Df | 40 | 38 | 3.8e[1] | 38 | 38 | 3.8e[1] | 38 |
| | p-value | 0.52 | **0.04** | 0.14 | **0.02** | 0.96 | 0.52 | 0.76 |
| $NH_4^+$-N | t | 0.06 | -2.74 | 2.51 | 1.85 | 0.88 | -0.50 | -0.24 |
| | Df | 40 | 37.53 | 38 | 38 | 36.68 | 38 | 38 |
| | p-value | 0.95 | **0.01** | 0.14 | **0.07** | 0.38 | 0.62 | 0.81 |
| $PO_4^{3-}$-P | t | 0.23 | -2.09 | -1.03 | 2.47 | -0.35 | 0.43 | 0.06 |
| | Df | 40 | 37.64 | 3.8e[1] | 38 | 36.81 | 3.8e[1] | 38 |
| | p-value | 0.82 | **0.04** | 0.31 | **0.02** | 0.73 | 0.67 | 0.95 |
| N:P ratios | t | -0.50 | 2.35 | -0.73 | -1.46 | 0.09 | -0.36 | 0.42 |
| | Df | 4 | 35.03 | 34.03 | 34 | 34.71 | 34.08 | 34 |
| | p-value | 0.65 | **0.03** | 0.47 | 0.15 | 0.93 | 0.72 | 0.67 |

### 4.4.4 Microbial biomass and inorganic N

Table 4-5 shows the summarised averages (mean±se) of gravimetric volume content, soil extractable $NH_4^+$-N and $NO_3^-$-N, microbial biomass C, N and P, and microbial biomass C:N, C:P and N:P ratios, per soil horizon, from the three soil sampling

campaigns (March, July and November 2018) at BIFoR FACE during Year 2 of $eCO_2$ enrichment.

Ambient arrays were drier than $eCO_2$ arrays across all three soil horizons in all three soil campaigns (Table 4-5). Specifically, O horizons were 2-5 times wetter than A and 2-5 times wetter than B horizon in ambient arrays, while O horizons were 3-4 times wetter than A and 4-6 times higher than B horizons in $eCO_2$ arrays. March and November had comparable moisture values, while July was the driest in both ambient and $eCO_2$ arrays. Soil extractable $NH_4^+$-N availability was higher in $eCO_2$ arrays for O horizons only (except in July), while for A and B horizons. $NH_4^+$-N availability was higher in ambient arrays. Specifically, O horizons had 2-5 times higher $NH_4^+$-N than A and 4-6 times than B horizon in ambient arrays, while O horizons had 3-5 times higher $NH_4^+$-N than A and 5-6 times higher than B horizons in $eCO_2$ arrays. July had the highest $NH_4^+$-N availability, followed by November and March in both ambient and $eCO_2$ arrays. Soil extractable $NO_3^-$-N availability was higher ambient across all soil horizons in all three soil samplings. Specifically, O horizons had 5-6 times higher $NO_3^-$-N than A and 10-18 times than B horizon in ambient arrays, while O horizons had 6-7 times higher $NO_3^-$-N than A and 19-36 times higher than B horizons in $eCO_2$ arrays. November had the highest $NO_3^-$-N availability, followed by March, while the lowest $NO_3^-$-N availability was observed in July in both ambient and $eCO_2$ arrays.

Microbial biomass C was higher in $eCO_2$ arrays in O and B horizons, whereas in A horizons, microbial biomass C was higher in ambient arrays (Table 4-5). Specifically, O horizons had 2-3 times more microbial biomass C than A and 3-13 times more than B horizon in ambient arrays. O horizons were 2-7 times more microbial biomass C than A and 6-11 times more than B horizons in $eCO_2$ arrays. July had the highest microbial biomass C, while March and November had comparable values in ambient

and $eCO_2$ arrays. Microbial biomass N had the same pattern as microbial biomass C, where N was higher in $eCO_2$ arrays in O and B horizons.

In contrast, in A horizons, microbial biomass N was higher in ambient arrays. Specifically, O horizons had 2-4 times more microbial biomass N than A and 2-12 times more than B horizon in ambient arrays. O horizons were 2-10 times more microbial biomass N than A and 3-9 times more than B horizons in $eCO_2$ arrays. March and November had comparable values; however, July had approximately 4 times higher microbial biomass N in ambient and $eCO_2$ arrays. Similarly, microbial biomass P was higher in $eCO_2$ arrays in O and A horizons (except March 2018, where microbial biomass P was higher in ambient arrays in A horizon), while B horizons were similar in ambient and $eCO_2$ arrays. Note that there is no microbial biomass P data available for July. Specifically, O horizons had 6-7 times more microbial biomass P than A and 9-23 times more than B horizon in ambient arrays. O horizons were 5-17 times more microbial biomass O than A and 15-45 times more than B horizons in $eCO_2$ arrays. March had higher microbial biomass P than November in both ambient and $eCO_2$ arrays.

Microbial biomass C:N ratios were lower in the top layer and increased by depth in March and November, whereas in July, the top layer had higher C:N ratios and decreased by depth, with ratios being slightly higher in $eCO_2$ arrays (Table 4-5). C:N ratios in March and November were comparable, while in July were approximately 2 times lower for O, 7 times lower for A, and 10 and 39 times lower for B horizon in ambient and $eCO_2$ arrays, respectively. Microbial C:P and N:P ratios increased with depth, with ambient arrays having higher C:P and N:P ratios in O horizons. March had higher C:P and N:P ratios in the top layer, while November had higher ratios at the deeper layers. Note that there is no C:P and N:P data available for July.

$eCO_2$ had no statistically significant effect on neither microbial biomass nor on inorganic N (see 4.8 Supplementary Material, Table S4-1). O horizons were statistically different from A and B horizons for all variables (gravimetric volume content, soil extractable $NH_4^+$-N and $NO_3^-$-N, microbial C, N, and P). In contrast, A and B horizons were statistically similar. Moreover, microbial ratios were statistically equivalent across all three soil horizons. Gravimetric volume content, soil extractable $NH_4^+$-N, and microbial biomass N were statistically different in March vs. July, while soil extractable $NH_4^+$-N, $NO_3^-$-N, microbial biomass N, and microbial C:N ratios were statistically different in July vs. November.

**Table 4- 5:** *Summary on concentrations of gravimetric volume content, soil extractable $NH_4^+$-N and $NO_3^-$-N, microbial biomass C, N and P, and microbial biomass C:N, C:P and N:P ratios (mean±se) from three soil sampling campaigns (March, July, and November 2018) per horizon under ambient ($aCO_2$) and $eCO_2$ conditions, during the Year 2 of $eCO_2$ enrichment at BIFoR FACE.*

| | Gravimetric volume content (%) | | Soil extractable $NH_4^+$-N (µg/g) | | Soil extractable $NO_3^-$-N (µg/g) | | Microbial biomass C (µg/g) | | Microbial biomass N (µg/g) | | Microbial biomass P (µg/g) | | Microbial C:N | | Microbial C:P | | Microbial N:P | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ | $aCO_2$ | $eCO_2$ |
| March 2018 | | | | | | | | | | | | | | | | | | |
| O | 59.1±9.5 | 81.6±18.1 | 6.6±2.1 | 7.8±1.0 | 20.0±4.1 | 18.9±2.3 | 652.5±61.0 | 822.5±151.2 | 197.3±21.4 | 254.7±58.7 | 60.2±17.7 | 85.3±25.3 | 3.4±0.3 | 3.5±0.3 | 77.6±66.0 | 53.3±34.4 | 21.5±18.0 | 14.3±8.6 |
| A | 25.5±2.6 | 21.5±1.2 | 2.4±0.3 | 1.8±0.4 | 4.6±1.1 | 2.6±0.5 | 180.6±38.7 | 115.0±12.3 | 50.3±13.8 | 26.6±3.8 | 10.2±1.8 | 5.0±1.0 | 4.0±0.4 | 4.6±0.4 | 46.8±26.9 | 74.1±50.6 | 13.5±8.1 | 22.2±17.0 |
| B | 16.2±0.5 | 21.3±4.9 | 1.9±0.2 | 1.4±0.2 | 1.9±0.3 | 1.0±03 | 92.7±19.8 | 106.4±31.9 | 16.5±2.7 | 29.5±10.9 | 2.6±0.9 | 1.9±0.7 | 5.9±0.9 | 4.0±0.3 | 86.9±36.2 | 219.9±148.9 | 17.2±7.6 | 67.0±49.2 |
| July 2018 | | | | | | | | | | | | | | | | | | |
| O | 21.3±1.7 | 36.2±2.3 | 31.2±12.2 | 22.3±5.1 | 13.8±0.9 | 12.0±1.9 | 1016.8±140.9 | 1500.8±303.2 | 850.3±110.3 | 987.0±201.9 | NA | NA | 1.3±0.2 | 2.3±0.8 | NA | NA | NA | NA |
| A | 11.7±0.5 | 12.6±1.4 | 6.2±0.7 | 4.9±0.6 | 2.8±0.6 | 2.0±0.6 | 309.9±94.0 | 284.4±57.4 | 441.5±38.1 | 443.9±35.4 | NA | NA | 0.7±0.2 | 0.6±0.1 | NA | NA | NA | NA |
| B | 9.7±0.5 | 8.6±0.4 | 4.8±0.4 | 3.3±0.4 | 0.9±0.4 | 0.4±0.1 | 76.9±32.0 | 131.9±12.9 | 376.3±12.9 | 288.8±50.9 | NA | NA | 0.2±0.1 | 0.6±0.2 | NA | NA | NA | NA |
| November 2018 | | | | | | | | | | | | | | | | | | |
| O | 47.1±8.4 | 74.5±14.0 | 7.5±2.2 | 12.1±4.5 | 35.4±8.2 | 25.7±4.7 | 630.0±128.5 | 1092.9±232.4 | 186.0±24.0 | 211.2±40.4 | 22.1±4.8 | 37.7±6.3 | 3.3±0.6 | 5.7±1.1 | 36.7±8.6 | 29.6±7.4 | 11.7±2.9 | 6.2±1.0 |
| A | 15.7±1.5 | 19.9±3.5 | 2.6±0.4 | 2.3±0.4 | 7.5±1.4 | 3.9±1.5 | 336.6±105.3 | 288.4±69.8 | 54.8±19.6 | 48.6±10.6 | 3.0±0.7 | 7.0±2.5 | 7.7±2.1 | 23.7±17.1 | 153.5±70.8 | 263.7±188.4 | 49.8±24.7 | 34.1±14.2 |
| B | 10.7±13.2 | 13.2±2.8 | 1.6±0.3 | 1.1±0.1 | 1.9±0.4 | 0.7±0.3 | 214.5±49.7 | 187.6±40.9 | 14.2±5.8 | 17.7±4.3 | 2.4±0.6 | 2.4±0.6 | 23.9±6.3 | 16.6±5.9 | 493.4±439.5 | 249.9±169.9 | 15.6±12.4 | 40.2±31.2 |

### 4.4.5 Year 1 vs. Year 2

A detailed description of the environmental conditions and belowground responses for Year 1 of $eCO_2$ enrichment can be found in *Chapter 3*. A brief description of the environmental conditions during both years can be found in Table 4-6 for accommodating comparisons. For this comparison, only the data from Year 1 and Year 2 were used, while Pre-treatment and Dormant were excluded, and they are presented as annual averages (±se). Year 2 had less Pr than Year 1. Moreover, VWC was higher in ambient arrays in Year 2 but lower in $eCO_2$ arrays than Year 1, while $T_s$ was lower during Year 2 in ambient and $eCO_2$ conditions. $R_s$ was lower during Year 2, with the decrease being more prominent in ambient arrays. Lastly, all three bioavailable nutrients had higher availabilities during Year 2, with the increase being more pronounced in $eCO_2$ arrays, specifically for $NO_3^-$-N.

However, $eCO_2$ had a statistically significant effect on $NO_3^-$-N availability in Year 1 (see *Chapter 3*) and $R_s$ in Year 2 (see section *4.4.1 $R_s$ response to environmental drivers and $eCO_2$*). Moreover, both VWC and $T_s$ in Year 2 were statistically different than Year 1, and the interactions between $eCO_2$ and year were statistically significant (see 4.8 Supplementary Material, Table S4-2).

**Table 4- 6:** *Environmental variables, $R_s$, and bioavailable nutrients (mean ± se) during the study period summarized in annual averages. As for Year 1 and 2, we denote only the $eCO_2$ enrichment period (Year 1, 3rd April – 27th October 2017 and Year 2, 26th March – 31st October 2018).*

| | Year 1 | | Year 2 | |
|---|---|---|---|---|
| **Variables** | **Ambient** | **$eCO_2$** | **Ambient** | **$eCO_2$** |
| Pr (mm) | 362.4 | | 358.1 | |
| VWC (%) | 10.6 ± 0.3 | 12.9 □ 0.4 | 11.2 ± 0.5 | 10.8 ± 0.5 |
| $T_s$ (°C) | 12.4 ± 0.2 | 12.7 ± 0.2 | 8.1 ± 0.2 | 8.5 ± 0.2 |
| $R_s$ (µmol $CO_2$ $m^{-2}$ $y^{-1}$) | 3.5 ± 0.1 | 4.3 ± 0.1 | 2.1 ± 0.1 | 3.2 ± 0.1 |
| $NO_3^-$-N (µg $NO_3^-$-N $cm^{-2}$ $d^{-1}$) | 10.5 ± 1.2 | 7.6 ± 0.8 | 17.9 ± 4.8 | 15.3 ± 4.9 |
| $NH_4^+$-N (µg $NH_4^+$-N $cm^{-2}$ $d^{-1}$) | 4.3 ± 0.8 | 4.3 ± 0.8 | 6.3 ± 1.7 | 6.4 ± 1.3 |
| $PO_4^{3-}$-P (µg $PO_4^{3-}$-P $cm^{-2}$ $d^{-1}$) | 1.0 ± 0.2 | 0.9 ± 0.2 | 2.0 ± 0.7 | 2.1 ± 0.8 |

Fig. 4-8 shows the annual $R_s$ estimates (mean±se) for Year 1 and 2 at BIFoR FACE. Year 1 annual $R_s$ was was 1,341 ± 21 g C $m^{-2}$ $y^{-1}$, while in $eCO_2$ arrays was approximately 21% higher (1,624 ± 24 g C $m^{-2}$ $y^{-1}$). Year 2, the annual $R_s$ was 714 ± 15 g C $m^{-2}$ $y^{-1}$, while in $eCO_2$ arrays was approximately 36.5% higher (973 ± 19 g C $m^{-2}$ $y^{-1}$). Ambient arrays had a 47% decrease during Year 2, while $eCO_2$ arrays had a 40% decrease.

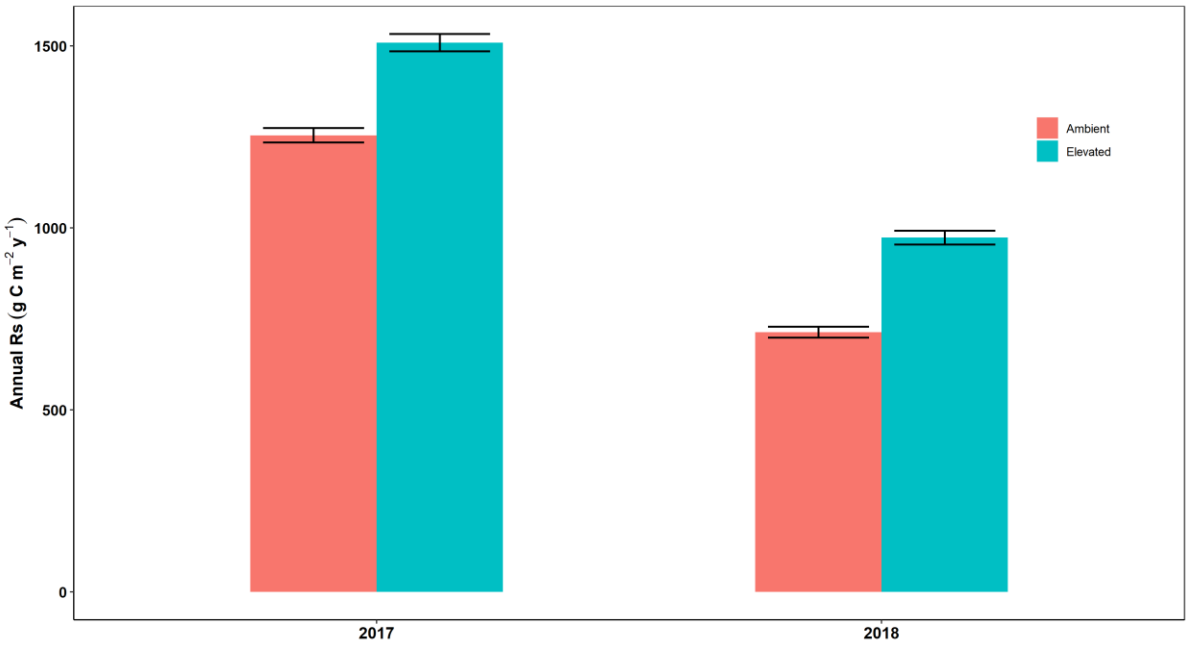


***Figure 4- 8:*** *Mean annual $R_s$ in g C $m^{-2}$ $y^{-1}$in ambient (orange) and $eCO_2$ (blue) arrays during the first two years at BIFoR FACE. The error bars denote the standard error.*

## 4.5 Discussion

Soils at BIFoR FACE exhibited strong seasonal patterns in soil moisture, temperature, and respiration during the second year of $eCO_2$ enrichment (Fig. 4-4). Year 2 of $eCO_2$ enrichment coincided with a series of extreme events (Beast from the East, winter moth defoliation event, 6-week heatwave), having a great impact on belowground responses at BIFoR FACE. $eCO_2$ had a statistically significant effect on soil respiration during Year 2 of $eCO_2$ enrichment (Table 4-1, p-value = 0.02), increasing by approximately 37% soil respiration on annual averages. However, no statistically significant differences were observed on bioavailable soil nutrients or microbial biomass (Table 4-3 and Table S4-1). $eCO_2$ had no statistically significant effect on any of the bioavailable nutrients; however, all three nutrients responded differently under $eCO_2$. $NO_3^-$-N decreased by 15% under $eCO_2$, $PO_4^{3-}$-P increased by 5% under $eCO_2$,

while $NH_4^+$-N had no difference between ambient and $eCO_2$ arrays on annual averages.

### 4.5.1 Soil respiration and environmental drivers

The annual estimates of soil respiration during the first year of $eCO_2$ enrichment were 714±15 vs. 973±19 g C $m^{-2}$ $y^{-1}$ in ambient and $eCO_2$ arrays. These estimates are within the range of observations (500-2,100 g C $m^{-2}$ $y^{-1}$) reported for similar temperate forests (Campbell and Law, 2005) under non-extreme events conditions. Our estimates show that $eCO_2$ could mitigate the extreme event's impact on an annual average and agree with studies that have monitored soil respiration responses under synthetic drought and heatwave conditions (Roy *et al.*, 2016).

The models used for the soil respiration estimates were the triple interaction between $eCO_2$, soil temperature, and moisture for both Dormant and Year 2. Both models predicted 73 and 60% of the variability (Table 4-2). Our modelling estimates are defensible since the correlation between predicted and observed soil respiration values was statistically significant in ambient and $eCO_2$ arrays. Both models chosen did not have the lowest AIC, but they had the highest $R_c^2$. Although our analysis indicates that the primary driver during the second year of $eCO_2$ enrichment was soil moisture, our analysis highlights the importance of soil temperature (Fig. 4-4, Table 4-2). When soil temperature was included in our mixed-model analysis, the model's predictive capability increased by 11% (49 vs. 60% predictive capacity, respectively). Moreover, this analysis's objective was to utilise the models with the greatest predictive capacity rather than parsimony to acquire robust estimates. Thus, for this study's objectives, the models chosen for imputing missing data for soil respiration,

although they were not of the highest parsimony, offered the best possible goodness of fit (Glen, 2015).

Soil moisture had very distinctive effects on soil respiration in ambient and $eCO_2$ arrays (Fig. 4-5a). As the soil water content increases in ambient arrays, soil moisture becomes a limiting factor for soil respiration. However, in $eCO_2$ arrays, soil moisture was observed to affect Rs across the entire soil moisture range positively. Moreover, soil temperature was a limiting factor for soil respiration in levels higher than 13 and 14 °C in ambient and $eCO_2$ arrays, respectively, with the effect being sharper in $eCO_2$ arrays (Fig. 4-5b). However, in the highest soil temperature ranges, soil moisture levels also contribute to the soil respiration response and vice versa; in the highest soil moisture levels, the soil temperature also contributes to the observed response. Thus, although investigating soil responses to environmental factors individually might provide a more straightforward approach, *in situ,* the environmental factors interact in a more complex and dynamic way with soil respiration.

It is generally observed that during drought periods, soil respiration is decreased (Goulden *et al.*, 1996; Fierer and Schimel, 2002; Harper *et al.*, 2005; Borken *et al.*, 2006; Garten, Classen and Norby, 2009; Muhr and Borken, 2009; Bloor and Bardgett, 2012; Selsted *et al.*, 2012; Balogh *et al.*, 2016; Sun, Lei and Chang, 2019; Bréchet *et al.*, 2019; Ficken and Warren, 2019) due to reduced photosynthesis (Högberg *et al.*, 2001; Ruehr *et al.*, 2009; Van der Molen *et al.*, 2011), reduced C transfer from plants to soil bacteria (Zak *et al.*, 1994; Fuchslueger *et al.*, 2014) and fungi (Talbot, Allison, and Treseder, 2008), and reduced microbial and mycorrhizal exoenzyme production (Kuzyakov and Domanski, 2000; Schimel and Weintraub, 2003; Wieder, Bonan and Allison, 2013). Moreover, drought can decrease soil respiration due to soil water deficit (Orchard and Cook, 1983; Skopp, Jawson and Doran, 1990; Howard and Howard,

1993; Jassal *et al.*, 2008; Manzoni, Schimel and Porporato, 2012) or by altering the soil respiration's sensitivity to soil temperature and uncouple soil moisture, temperature, and respiration by reduction of soluble C substrate, extracellular enzyme activities and microbial processes (Selsted *et al.*, 2012; Wang *et al.*, 2014).

Seasonal temperature sensitivity $Q_{10}$ was 1.40 in ambient vs. 1.23 in $eCO_2$ arrays during Year 2 at BIFoR FACE, which is lower than other forests experiencing dry conditions varying from 1.9-4.8 (Rey *et al.*, 2002; Janssens and Pilegaard, 2003; Curiel Yuste *et al.*, 2004; Borken *et al.*, 2006; Davidson, Janssens and Lou, 2006). This indicates that soil respiration in ambient arrays was more sensitive to soil temperature than in $eCO_2$ arrays. The low $Q_{10}$ values observed during Year 2 of $eCO_2$ enrichment do not necessarily represent the temperature sensitivity differences. When $Q_{10}$ is calculated as an annual/seasonal value, it is influenced strongly by the magnitude of the soil respirations seasonal changes (Curiel Yuste *et al.*, 2004). Moreover, under severe drought stress, the soil temperature is not a reliable soil respiration predictor; thus, $Q_{10}$ calculations are challenging (Moncrieff and Fang, 1999). Lastly, annual/seasonal $Q_{10}$ values do not only represent the changes in soil temperature but also changes in other seasonally oscillating variables and processes, such as soil moisture, litter inputs, microbial populations, and root biomass (Curiel Yuste *et al.*, 2004).

Previous FACE experiments in forest ecosystems have observed different magnitudes of increase (Duke: 17.4%; FACTS-I: 39.2%, FACTS-II: 49.4% for *P. tremuloides* and 59.5% for *Betula/Populus*; POPFACE: 46.7% for *P. eur*, 42.3% for *P.alba* and 34.2% for *P. nigra*, ORNL: 11.2%) in soil respiration rates during their second year of $eCO_2$ enrichment (King *et al.*, 2004; Bernhardt *et al.*, 2006). One needs to bear in mind two crucial points when comparing the $eCO_2$ induced increase observed in other FACE

experiments in the past. Firstly, all these forest ecosystems were either plantations or young forests on disturbed soils. Secondly, none of those experiments experienced various extreme events as BIFoR FACE during their second year of operation. EucFACE is the only FACE experiment conducted in mature woodland, and during their second year of $eCO_2$ enrichment, they observed a 9% increase in soil respiration rates (Drake *et al.*, 2018). Secondly, none of these experiments experienced such an ecosystemic perturbation during their second year of operation as BIFoR FACE.

Although the Beast from the East event technically happened before the switch-on of eCO2 enrichment during the second year of eCO2 enrichment at BIFoR FACE, we cannot exclude the biological importance of such an event on an ecosystemic level. The low temperatures and snowfall lasted until late March 2018, and the $eCO_2$ switch-on date was on 26th March. The freezing-thawing of soil leads to pulses of soil respiration (Goulden *et al.*, 1998; Larsen, Jonasson and Michelsen, 2002; Schimel, Bilbrough and Welker, 2004; Kurganova, Teepe and Loftfield, 2007) by making available decomposable organic C via microbial death (Herrmann and Witter, 2002) and thus, nutrient release. Moreover, upon thawing, organic material damaged due to frost becomes available for decomposition (Priemé and Christensen, 2001).

Another critical aspect to consider is that the Beast from the East might have triggered the moth infestation outbreak. Oak budburst was on 20th April, and the first leaf was on 25th April (approximately 20 days later than 2017). Winter moth (*Operophtera brumata*) egg hatching and the synchronised relationship with oak bud burst has been well documented (Feeny, 1970; Dongen *et al.*, 1997). The egg hatching is predominantly triggered by abiotic cues (temperature). However, for the past 30 years, a clear mistiming has been recorded between winter moth egg hatching and budburst due to changes in the weather pattern rather than increases in spring temperatures

(Visser and Holleman, 2001). Moreover, such changes in temperature patterns create asynchrony in multiple levels of the trophic chain (Visser *et al.*, 1998; Stevenson and Bryant, 2000) that can also affect the predators of winter moths, such as great tits (*Parus major*). Thus, Beast from East might have caused a delay in winter moth and great tits reproduction cycles, triggering the defoliation event.

Defoliation events have been observed to strongly increase soil respiration due to as a result of labile organic C and N inputs in the soil through frass, leaf input, and incest cadavers, which in turn increases available substrate for microbial decomposition (Lovett and Ruesink, 1995; Christenson *et al.*, 2002; Hillstrom *et al.*, 2010; I-M-Arnold *et al.*, 2016; Grüning *et al.*, 2018). However, when the defoliation events coincide or are succeeded by dry conditions, microbial decomposition can be inhibited (Jung and Lunderstadt, 2000). Moreover, $eCO_2$ has been observed to alter plants' chemical composition by increasing the tannin concentrations and the C/N ratios in leaves, thus reducing the leaf litter quality (Kopper and Lindroth, 2003). Accordingly, altered leaf chemical composition will alter frass composition (Madritch, Donaldson and Lindroth, 2007). That could have a severe impact on belowground processes since condensed tannins can reduce nutrient availability, decomposition rates and alter soil respiration (Parsons, Lindroth and Bockheim, 2004; Madritch, Jordan and Lindroth, 2007; Parsons, Bockheim and Lindroth, 2008; Schweitzer *et al.*, 2008).

Studying one extreme event individually on an ecosystemic level is challenging due to the lack of warning before an event and experimental data, let alone three extreme events with intertwined time barriers. Therefore, we suggest that the overall decreased soil respiration response during Year 2 was driven by the combination of extreme events, with $eCO_2$ dampening the extreme events' effect.

### 4.5.2 Bioavailable inorganic N and P

$eCO_2$ had no statistically significant effect on soil biota available nutrients during Year 2 of $eCO_2$ enrichment at BIFoR FACE. All three soil bioavailable nutrients ($NH_4^+$, $NO_3^-$ and $PO_4^{3}$) followed the same seasonal pattern; the availability of soil nutrients increased during spring/early summer, with a steep decrease in mid-summer, followed by a five- and eightfold ($NH_4^+$, $PO_4^{3-}$ and $NO_3^-$, respectively) increase during autumn. Soil moisture and precipitation were found to have a statistically significant effect on soil nutrient availability, whereas soil temperature had no statistically significant effect.

Drought has been observed to slow plant growth by reducing N and P uptake, transport, and redistribution (Rouphael *et al.*, 2012), limiting ion mobility in the soil and microbial activity (Kreuzwieser and Gessler, 2010; Cregger *et al.*, 2014). Moreover, limited N and P mineralisation and inorganic N fluxes have been associated with drought events (Sardans and Peñuelas, 2004; Borken and Matzner, 2009). Although there was a substantial decrease in bioavailable nutrients in the middle of the season, we cannot claim that this observation accurately reflects the actual availability since ion exchange membranes require good contact with the soil to transfer from soil water successfully. Under such low soil moisture conditions, it is possible that the necessary soil-membrane contact was not achieved.

Following the drought event, all three soil nutrients' availability increased substantially as the rainfall increased both in intensity and frequency. Lengthy periods of drought have been linked to an accumulation of soil nutrients which subsequently are made available to soil fauna after soil rewetting (Fierer and Schimel, 2002). Moreover, soil rewetting post-drought causes a boost in decomposition and C and N mineralization (Birch, 1964), thus increasing the soil nutrient availability ephemerally. Although drying-rewetting episodes have been observed to decrease N losses in forests (Muhr,

Franke and Borken, 2010), if rainfall events intensified, N losses due to denitrification can occur (Dick, Skiba and Wilson, 2001).

### 4.5.3 Microbial biomass and inorganic N

Microbial biomass C and N were higher under $eCO_2$ conditions in all three seasons, with the highest recorded during summer, albeit not statistically significant. These results are in agreement with previous studies (Lipson, Wilson and Oechel, 2005). Microbial biomass C and N (data not available for microbial biomass P) exhibited their highest concentrations during the summer campaign. These findings agree with previous studies, where under combined $eCO_2$ and drought conditions, the microbial biomass C and N were more elevated than under drought or $eCO_2$ alone (Xue *et al.*, 2017). In our study, the mean annual microbial biomass C:N, C:P, and N:P ratios were 2.67, 57.15, and 16.6, respectively, under ambient conditions. Under $eCO_2$ conditions, the mean annual microbial biomass C:N, C:P, and N:P ratios were 3.7, 41.45, and 10.25, respectively. It has been observed that the microbial biomass C:N, C:P, and N:P ratios for forests are 8.2, 74, and 8.9, respectively (Cleveland and Liptzin, 2007), while the global microbial biomass C:N, C:P, and N:P ratios for temperate broadleaf forests specifically are 8.3, 46 and 4.4 (Xu, Thornton and Post, 2013).

Drought is generally observed to constrain the accumulation of microbial biomass (Zhang and Zak, 1998) due to osmotic stress resulting in cell lysis and microbial death (Turner *et al.*, 2003), and alterations in belowground inputs in the rhizosphere as a result of plant adaptation to drought stress (Milcu, Paul and Lukac, 2011). On the other hand, the $eCO_2$ effect is more convoluted since there is contradictory evidence of positive, negative, and no effect (Zak *et al.*, 2000; Freeman *et al.*, 2004). Even more

so, the evidence of the interactive effects of $eCO_2$ and drought on belowground microbial processes is rather limited (Kassem *et al.*, 2008).

Moreover, following a defoliation event, C/N rations have been observed to decrease due to large inputs of labile C and N through frass, leaf material, and insect cadavers (Kaukonen *et al.*, 2013; I-M-Arnold *et al.*, 2016; Brouillard *et al.*, 2017; Mikkelson *et al.*, 2017; Grüning *et al.*, 2018). Both C and N inputs during insect outbreaks are highly water-soluble. Thus they can easily and promptly penetrate deeper soil horizons (Zimmer and Topp, 2002; le Mellec, Habermann and Michalzik, 2009). N inputs via frass and fresh leaf material have been observed to increase by 300% (Grüning *et al.*, 2017) as well as the lower C/N inputs via fresh leaf material, as opposed to senescent leaf input (Kopáček et al., 2010), might explain the observed decrease in July spring C/N ratios at BIFoR FACE during the second year of $eCO_2$ enrichment. However, one needs to be cautious when interpreting the summer soil sampling microbial biomass C:N ratios, where the ratios are observed to be lower than 3 (for the O horizons). Even though the absolute ranges of the elemental concentrations in microbial biomass can vary greatly during the different seasons, the element ratios are well-constrained (Cleveland and Liptzin, 2007). The C:N ratios for bacteria are around 5.5, whereas for fungi are 8.3, ratios of 1 and 2 are stoichiometrically impossible (Scow, 1997).

### 4.5.4 Year 1 vs. Year 2

Figure 4-9 shows the percentage change (mean ± se) of soil variables between ambient and $eCO_2$ conditions the first two years of $eCO_2$ enrichment at BIFoR FACE. $R_s$ was higher under $eCO_2$ for both Year 1 and Year 2 (~21 and 36%, respectively), and the change was more prominent in Year 2. The temperature sensitivity $Q_{10}$ was

decreased by 30 and 12% for Year 1 and 2, respectively, under $eCO_2$. Bioavailable $NO_3^-$-N was decreased by 27 and 15% under $eCO_2$ during Year 1 and 2, respectively, while $NH_4^+$-N had no difference. Moreover, $PO_4^{3-}$-P had a 10% decrease during Year 1 and a 5% increase during Year 2 under $eCO_2$. Furthermore, there was a ~50 and 8% increase in microbial biomass C in the O and B horizons, respectively, whereas there was a 15% decrease in microbial biomass C in A horizons under $eCO_2$. Under $eCO_2$ microbial biomass, N increased by 15% in the O horizon but decreased by 2 and 17 at A and B horizons. A similar pattern was observed for microbial biomass P, with the O horizons having a 53% increase under $eCO_2$, while there was an 8 and 16% decrease in A and B horizons. Lastly, both, soil inorganic $NH_4^+$-N and $NO_3^-$-N decreased under $eCO_2$, with the decrease being intensified with depth.

Ultimately, $R_s$ during Year 2 was decreased by 47 and 40% under ambient and $eCO_2$ conditions, respectively, compared to Year 1. Contrastingly, all soil bioavailable nutrients' concentrations were higher during Year 2 under both ambient and $eCO_2$ conditions. $NH_4^+$-N availability increased by 47 and 49% under ambient and $eCO_2$ conditions, respectively. Similarly, $NO_3^-$-N availability increased by 70 and 101% under ambient and $eCO_2$ conditions, respectively, during Year 2. Sequentially, $PO_4^{3-}$-P availability during Year 2 increased by 100 and 133% under ambient and $eCO_2$ conditions, respectively.

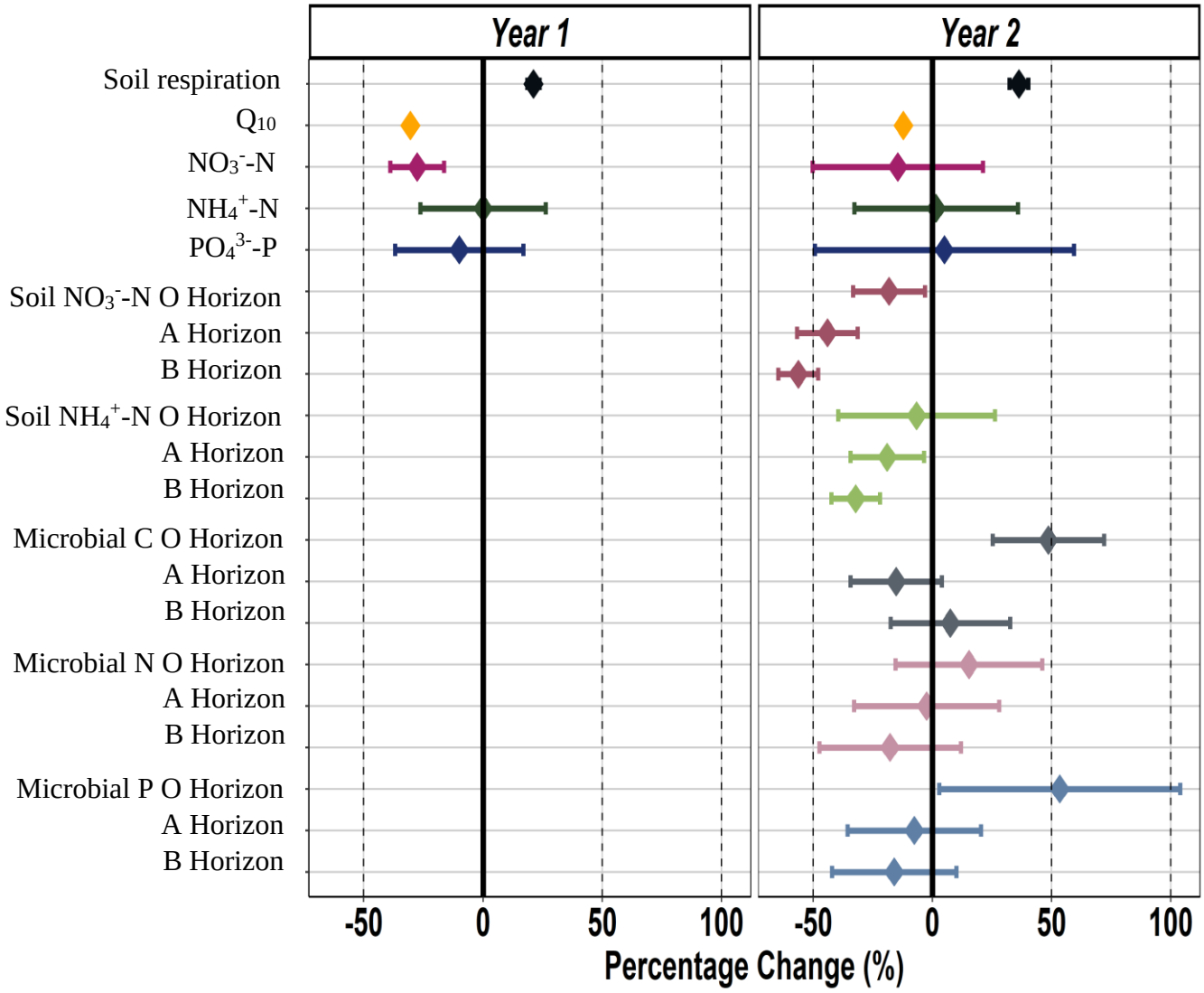


***Figure 4- 9:*** *Percentage change of $R_s$, soil nutrient availability (bioavailable and inorganic N), microbial biomass C, N, and P (per soil horizon) between ambient and $eCO_2$ conditions at BIFoR FACE the first two years of $eCO_2$ enrichment. The error bars denote standard error. Note that data for inorganic N and microbial biomass C, N, and P are available only for Year 2*

### 4.5.5 Interactions of carbon and nutrient cycling

In this study, we had the opportunity to observe the effects of extreme events in a mature temperate woodland under $eCO_2$. We hypothesise that the substantial decrease in soil respiration observed during the second year of $eCO_2$ enrichment is plausible to be attributed to water deficit due to the prolonged heatwave. Moreover,

the late summer/early autumn substantial increase in nutrient availability can be attributed to the winter moth infestation event, where all the easily decomposable material deposited on the soil floor due to the infestation became available for decomposition with the increase of precipitation, which in turn increased the soil respiration.

Our data showcase that carbon and nutrient dynamics are affected differently by the different extreme events. The heatwave had a substantial impact in both carbon and nutrient cycling; however the moth outbreak, in the short-term, had a greater impact on nutrient availability rather than soil respiration. Large amounts of nutrients became available by the end of autumn, a period when the nutrient demand is not high. Although an increase in soil respiration was observed when nutrient availability was high, this was not reflected in the annual averages. Thus, the carbon cycle was dominated by the heatwave whereas the winter moth outbreak dominated the nutrient cycle.

### 4.5.6 Future implications

Extreme events are predicted to increase in the future (IPCC, 2007) due to climate change. $eCO_2$ increase is happening in a step-wise change, and its effect can be assumed constant, whereas the extreme events happen abruptly, and thus their impact is more severe. This is reflected in our observations in this study, where the series of extreme events dominated the belowground processes with $eCO_2$ dampening the magnitude of the extreme events. Moreover, combined extreme events tend to have less impact than the impact that the same events would have had individually (Raymond *et al.*, 2020).

Globally, droughts are predicted to dominate the terrestrial carbon cycle (Frank *et al.*, 2015). Severe drought conditions under $eCO_2$ can reduce the sap flow and canopy conductance, thus impacting the carbon gain (Warren, Norby and Wullschleger, 2011). Forest areas across the temperate region are predicted to be substantially negatively impacted by droughts (Schwalm *et al.*, 2010; Seneviratne *et al.*, 2012). Thus, severe drought might alleviate the $eCO_2$ fertilisation effect during extreme events. If trees cannot uptake the extra carbon and nutrient availability is limited, pausing severe impacts on the current forest carbon sink.

Moreover, there are considerable knowledge gaps in the physiological responses to pests on an ecosystemic scale (Kallarackal and Roby, 2012), let alone the multiple stressors combined effects. Although nutrient availability increases after a defoliation event, the time of the year and the local conditions are crucial for the nutrients' fate. Should nutrients become available during low demand time of the year and increased precipitation then the nutrient losses through leaching will increase since plants and microorganisms cannot utilise the extra amounts. Increased nutrient losses can lead to soil depletion, thus halting the extra carbon uptake.

## 4.6 Conclusions

In this study, we had the unprecedented opportunity to observe a parade of extreme events on a large-scale climatic experiment. The second year of $eCO_2$ enrichment at BIFoR FACE coincided with an unusually cold early spring, a moth outbreak causing a significant defoliation event, and a prolonged summer heatwave. eCO2 had a significant positive effect on soil respiration relative to ambient; however, no statistically significant increase was observed in microbial biomass. Soil respiration

was suppressed by the drought, while the moth outbreak dominated the nutrient cycling. The nutrient inputs increased on the forest floor via frass, caterpillar cadavers, and fresh leaf material once the water availability increased post-drought through increased precipitation episodes.

## 4.7 References


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## 4.8 Supplementary material

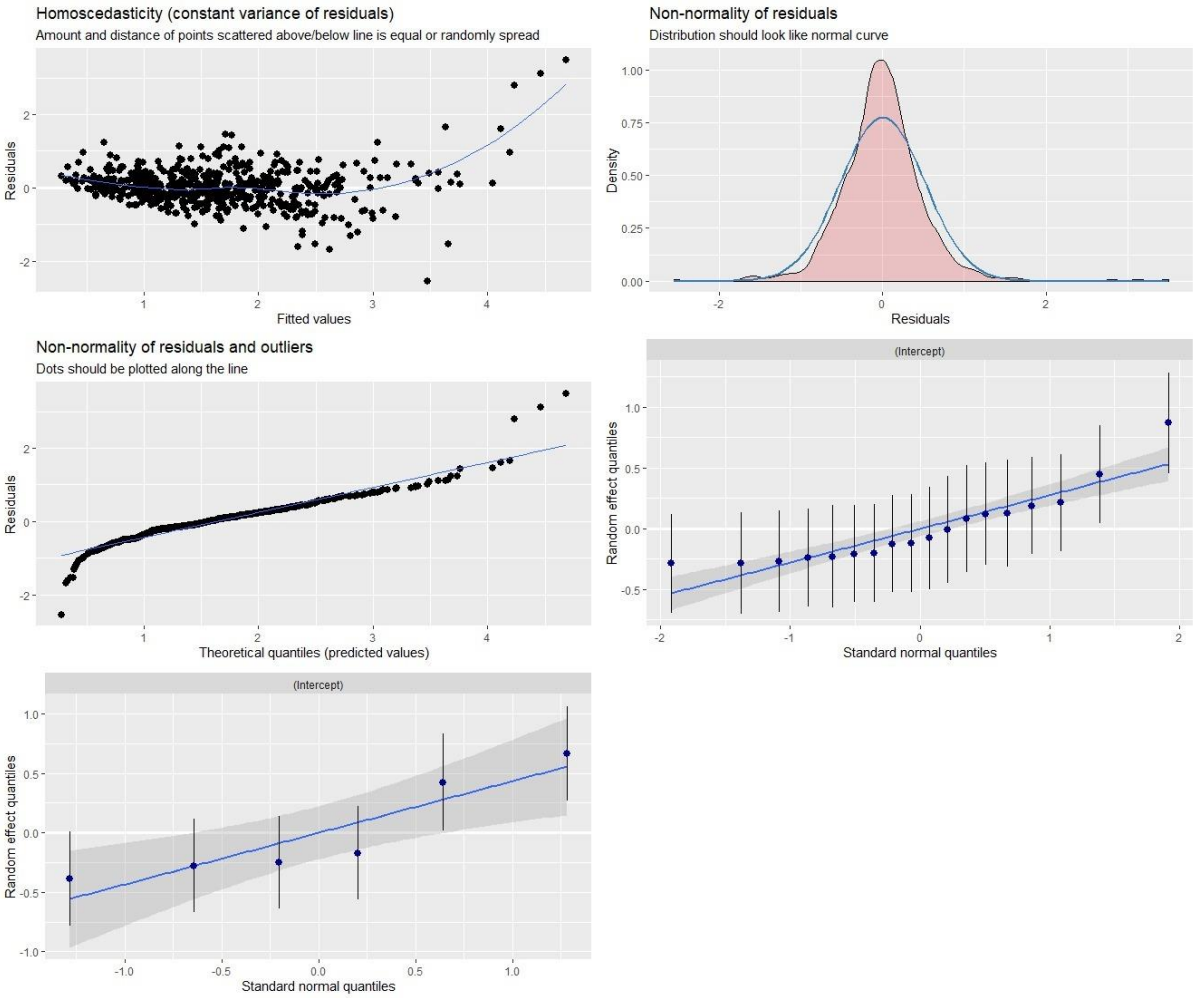


***Figure S4- 1:*** *Model validation for estimating soil respiration during Dormant for homoscedasticity, normality of residuals, and outliers and random effects.*

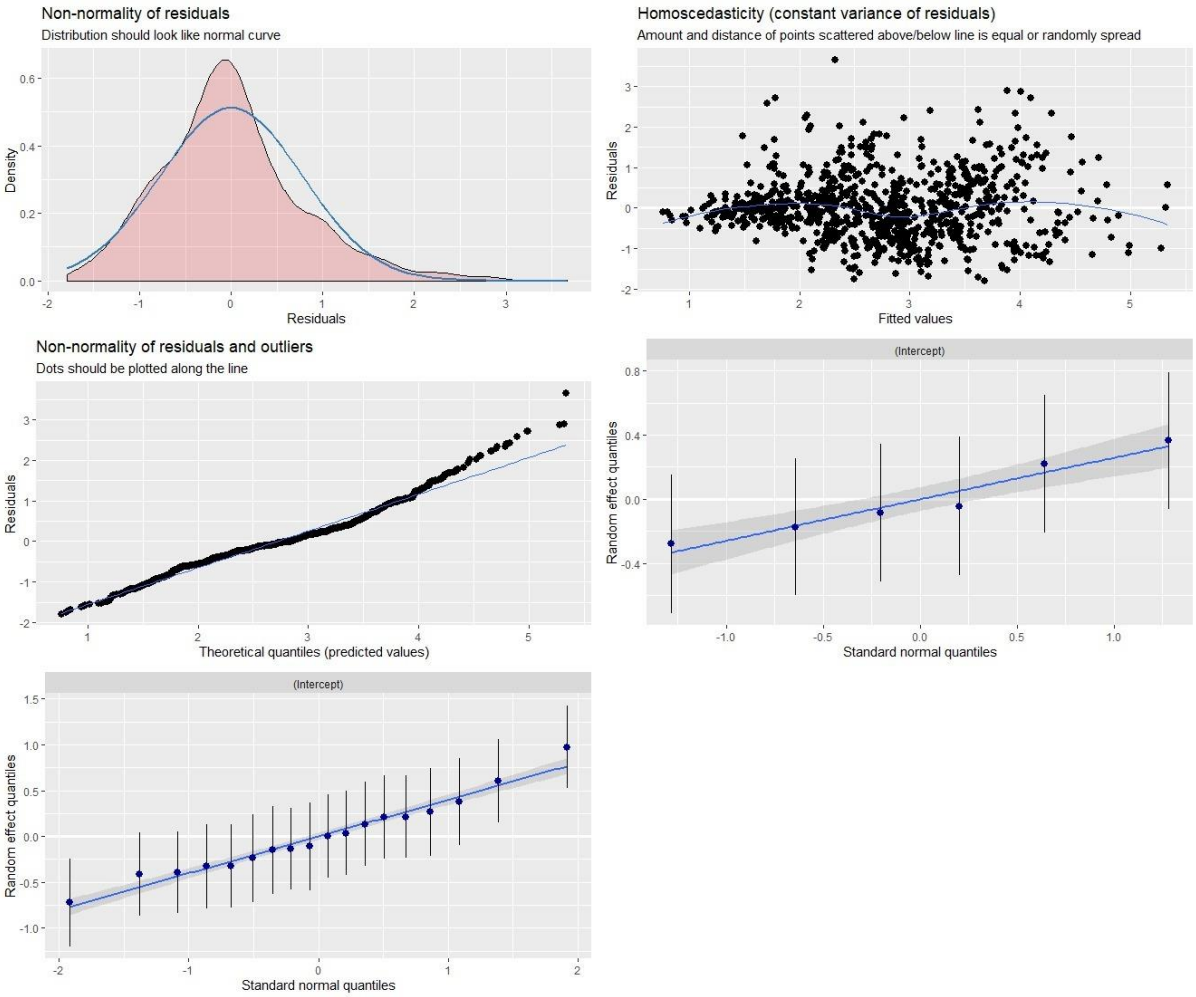


***Figure S4- 2:*** *Model validation for estimating soil respiration during Year 2 for homoscedasticity, normality of residuals, and outliers and random effects.*

Figure S4-3 shows the correlations between soil nutrient availability ($NO_3^-$-N Fig. S4-3a; $NH_4^+$-N Fig. S4-3b; and $PO_4^{3-}$-P Fig. S4-3c) and N:P ratios (Fig. S4-4d), and VWC, $T_s$ and Pr at BIFoR FACE during Year 2 of $eCO_2$ enrichment. The highest availability for all three nutrients was observed under conditions of high precipitation low VWC and $T_s$>12 °C. This indicates that Pr was a main driver during the warmer and drier period of Year 2 of $eCO_2$ enrichment at BIFoR FACE.

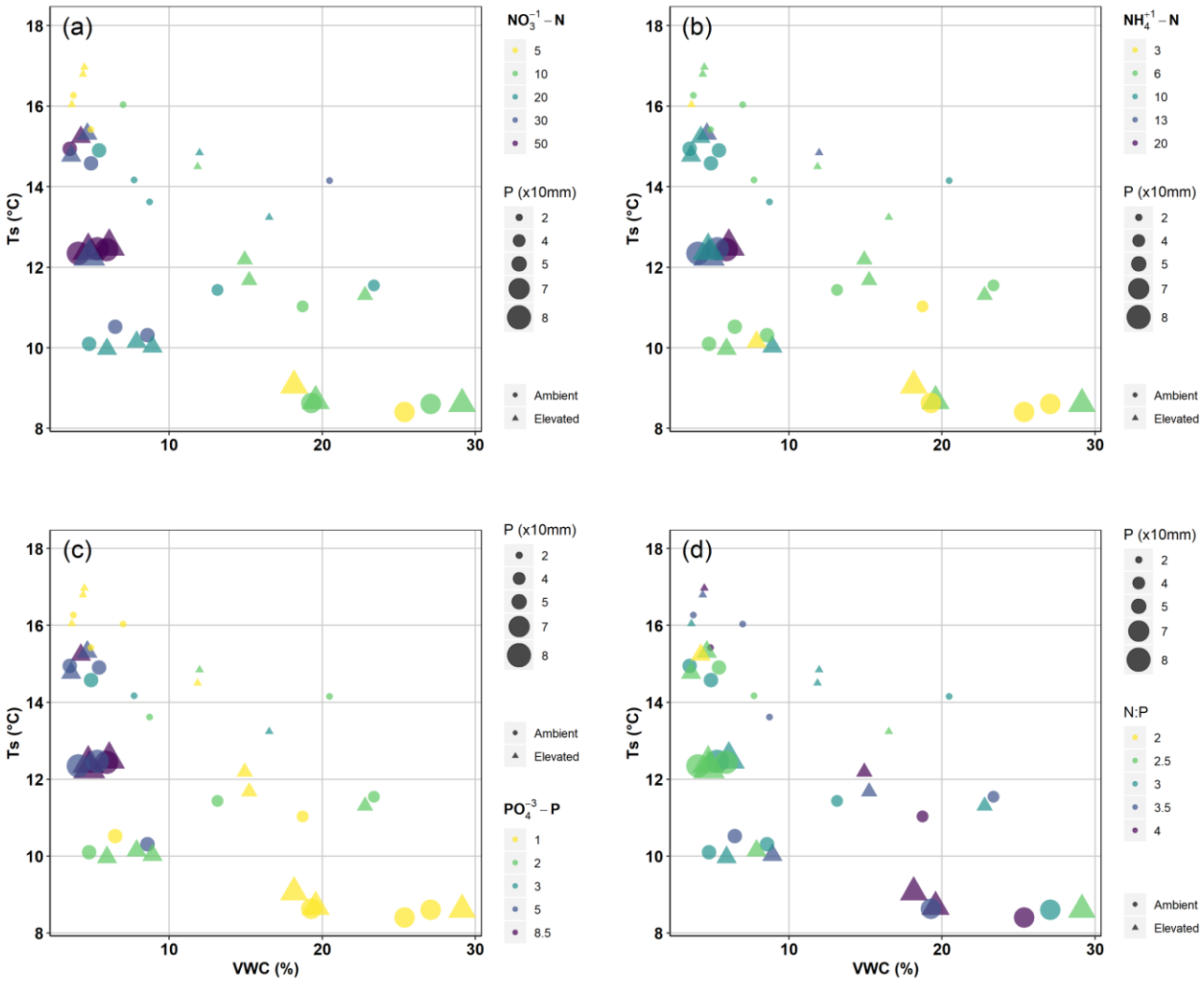


***Figure S4- 3:*** (a) $NO_3^-$-N, (b) $NH_4^+$-N, (c) $PO_4^{3-}$—P availability and (d) N:P ratios in correlation with VWC, $T_s$ and P at BIFoR FACE during Year 2 of $eCO_2$ enrichment. The triangles reflect the $eCO_2$ conditions while the circles reflect the ambient conditions. The colour luminance reflects the availability of soil nutrients and N:P ratios, which turns darker as the nutrient availability increases. The size of the shapes reflect the monthly cumulative precipitation in *10 mm, which increases as the precipitation increases.

**Table S4- 1:** *Summary of mixed-effects models of the $eCO_2$, soil horizons, and sampling campaign during Year 2 of $eCO_2$ enrichment on gravimetric volume content, soil extractable $NH_4^+$-N, soil extractable $NO_3^-$-N, microbial biomass C, N and P and microbial C:N, N:P and C:P ratios. P < 0.1 is shown in bold.*

| | | Covariates | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Response variables | | $eCO_2$ | Horizons O-A | Horizons O-B | Horizons A-B | Spring-Summer | Spring-Autumn | Summer-Autumn |
| Gravimetric volume content | t | 1.204 | 4.069 | 4.950 | -0.881 | -2.532 | 1.189 | -1.343 |
| | df | 4 | 152 | 152 | 152 | 152 | 152 | 152 |
| | P | 0.295 | **0.0001** | **<0.001** | 1 | **0.034** | 0.704 | 0.538 |
| Soil extractable $NH_4^+$-N | t | -0.401 | 3.751 | 4.061 | -0.310 | 3.257 | -0.094 | 3.163 |
| | df | 4 | 152 | 152 | 152 | 152 | 152 | 152 |
| | P | 0.709 | **0.0005** | **0.0001** | 1 | **0.003** | 1 | **0.005** |
| Soil extractable $NO_3^-$-N | t | -1.057 | 7.765 | 9.218 | -1.452 | -0.920 | -1.858 | -2.778 |
| | df | 4 | 152 | 152 | 152 | 152 | 152 | 152 |
| | P | 0.350 | **<0.001** | **<0.001** | 0.439 | 1 | 0.190 | **0.02** |
| Microbial biomass C | t | 1.264 | 4.719 | 5.976 | -1.275 | 1.506 | -0.649 | 0.863 |
| | Df | 150 | 146 | 146 | 146 | 146 | 146 | 146 |
| | P | 0.208 | **<0.001** | **<0.001** | 0.607 | 0.396 | 1 | 1 |
| Microbial biomass N | t | 0.294 | 2.862 | 3.242 | -0.439 | 6.988 | 0.019 | 6.799 |
| | Df | 154 | 150 | 150 | 150 | 150 | 150 | 150 |
| | P | 0.769 | **0.013** | **0.004** | 1 | **<0.001** | 1 | **<0.001** |
| Microbial biomass P | t | 1.071 | 3.379 | 3.795 | -0.422 | - | 1.389 | - |
| | Df | 104 | 100 | 100 | 100 | - | 102 | - |
| | P | 0.288 | **0.002** | **0.0004** | 1 | - | 0.168 | - |
| Microbial C:N ratio | t | 0.561 | -0.414 | -1.727 | 1.320 | -0.979 | -1.680 | -2.545 |
| | Df | 142 | 138 | 138 | 138 | 138 | 138 | 138 |
| | P | 0.576 | 1 | 0.253 | 0.560 | 0.982 | 0.279 | **0.033** |
| Microbial N:P ratio | t | 1.063 | -0.192 | -0.001 | -0.185 | - | -0.100 | - |
| | Df | 95 | 91 | 91 | 91 | - | 93 | - |
| | P | 0.290 | 1 | 1 | 1 | - | 0.921 | - |
| Microbial C:P ratio | t | 0.195 | -0.298 | -1.498 | 1.231 | - | -1.280 | - |
| | Df | 95 | 91 | 91 | 91 | - | 93 | - |
| | P | 0.845 | 1 | 0.402 | 0.654 | - | 0.204 | - |

***Table S4- 2:*** *Analysis of variance results for VWC and $T_s$ during Year 2 at BIFoR FACE. p-value< 0.1 is shown in bold.*

| Variables | $DF_{num}$ | $DF_{den}$ | F-value | P-value |
|---|---|---|---|---|
| *VWC* | | | | |
| $eCO_2$ | 1 | 4.0 | 0.375 | 0.574 |
| Year | 2 | 4076.4 | 250.675 | **<0.001** |
| $eCO_2$ * Year | 2 | 4076.4 | 47.960 | **<0.001** |
| $T_s$ | | | | |
| $eCO_2$ | 1 | 4.3 | 1.511 | 0.282 |
| Year | 2 | 4265.4 | 179.933 | **<0.001** |
| $eCO_2$ * Year | 2 | 4265.4 | 2.406 | **0.090** |

# Chapter 5: A double entendre challenge - Partitioning the components of soil respiration in a mature temperate woodland under $eCO_2$

## 5.1 Abstract

Soil respiration is the second-largest C flux after photosynthesis; however, the partitioning of soil respiration to biogenic sources remains difficult. The correct quantification of the contribution of its components to total soil respiration is of pivotal importance not only for the ecosystem's potential to uptake carbon but also to assess the carbon's fate within the ecosystem. Furthermore, disentangling total soil respiration to components contribution and thus to $CO_2$-derived sources and their responses to abiotic factors will improve the high uncertainty associated with the climatic models' carbon cycle. Here, we attempted to quantify the soil respiration to four respiratory components (heterotrophic, autotrophic, and the further partition to root and hyphal respiration) during the first two years of Free Air CO2 Enrichment (FACE) at a mature temperate woodland (BIFoR FACE). We observed that $eCO_2$ stimulated all respiratory components, except for root respiration, during the experiment's first year. This contribution was persistent during the second year through a series of extreme events (winter moth defoliation event, heatwave). The heterotrophic component was the main contributor to soil respiration in both years (over 70 and 80% during Year 1 and 2, respectively). However, the respiratory components' sensitivities to abiotic factors (soil temperature and moisture) were altered under the combined effects of $eCO_2$ and extreme events.

## 5.2 Introduction

The global average atmospheric $CO_2$ concentration in 2019 was 411.44 ppm and is the highest recorded in the past 800,000 years (https://www.esrl.noaa.gov/gmd/ccgg/trends/). Both the magnitude and the scale of the estimated atmospheric $CO_2$ increment have been suggested to depend on human-induced emissions, as well as on the efficiency with which ecosystems assimilate carbon (C) and store it (UNFCCC, 2005; Friedlingstein *et al.*, 2006; Friedlingstein *et al.*, 2014). Land vegetation and soil, collectively forming the terrestrial biosphere, are crucial components in the C biogeochemical cycle, currently amassing a mean of 450 Pg C as biomass stocks (Erb *et al.*, 2018). Thus, changes in the atmospheric $CO_2$ concentrations might have a potentially significant effect on C storage in the terrestrial biosphere due to either release or sequestration of C (Houghton, 2007).

Soil respiration ($R_s$), the $CO_2$ efflux from the forest soil, combines autotrophic root activity and associated rhizosphere organisms, heterotrophic bacteria and fungi, and soil fauna activity. $R_s$ is divided into autotrophic respiration ($R_a$), which is the $CO_2$ produced from the metabolic activity for root growth and maintenance; and heterotrophic respiration ($R_h$), which is the $CO_2$ produced from the decomposition of organic matter used as an energy source for microbial communities (Savage *et al.*, 2013). $R_a$ can be further partitioned to ectomycorrhizal (EM) fungi respiration ($R_m$) and root respiration ($R_r$). Although the analytical techniques used for assessing $R_s$ have been improved (Raich and Schlesinger, 1992; Davidson *et al.*, 2002; Baldocchi, 2003), there is still little information about the individual $CO_2$ emissions of the respiratory components, mainly due to the difficulty in separating the root from the EM, *in situ*, thus leading to overestimation of the $R_a$ and underestimations of the $R_h$ (Tomè *et al.*, 2016). Moreover, temporal sampling limitations, high-cost of measurement

instruments (Savage, Davidson, and Richardson, 2008), and $R_s$'s high variability both temporally and spatially (Bond-Lamberty and Thomson, 2010), add significant uncertainty to global C cycle models.

$R_s$ accounts for 60-90% of total ecosystem respiration (Longdoz, Yernaux, and Aubinet, 2000) and is the second-largest flux of C between terrestrial ecosystems and the atmosphere (Raich and Schlesinger, 1992; Zhao *et al.*, 2017). Nevertheless, $R_s$ is one of the least-studied elements of the terrestrial C cycle due to the challenges faced in spatial and temporal sampling and the high-cost measurement instruments (Savage, Davidson and Richardson, 2008). However, each soil component (roots, fungi, free-living microbes, and soil animals) has a unique dependence on substrate quality and abiotic factors and may exhibit different behaviours under a change in their environment or climate change (Wei, Weile and Shaopeng, 2010; Ma *et al.*, 2014). Thus, it is pivotal to successfully quantify the flux from each component, better understand the drivers and the processes involved, and make more accurate model predictions.

Over the last thirty years, the terrestrial biosphere provided a net sink for approximately 20% of the $CO_2$ emitted by anthropogenic emissions (Quéré *et al.*, 2018), with the forest ecosystems dominating the C storage (Pan *et al.*, 2011). Forest ecosystems play a significant role in the global C cycle since 80% of all aboveground terrestrial C, and 40% of belowground C are stored in forest biomass (Goodale *et al.*, 2002). Specifically, old-growth forests can store the sequestered C at exceptionally high levels compared to young and mature forests (Keith, Mackey and Lindenmayer, 2009; Keeton *et al.*, 2011; Burrascano *et al.*, 2013; McGarvey *et al.*, 2015), thus playing a crucial role in climate mitigation (Keeton, 2019). However, old-growth forests' response under elevated atmospheric $CO_2$ ($eCO_2$) yet remains uncertain. Although

$eCO_2$ has been observed to increase tree growth rates in young forests (Kallarackal and Roby, 2012), no study has been conducted in mature or old-growth forests, except for the EucFACE experiment in Australia. Growth responses due to $eCO_2$, and subsequently forest's capacity to sequester and store C long-term, might be constrained by environmental factors, such as soil moisture and soil nutrient availability (Keeton, 2019). Since the extent to which $eCO_2$ has transient or long-lasting effects on the factors mentioned above is yet uncertain, the question about the carbon storage capacity within old-growth forests under climate change is yet to be answered.

In the present study, we assessed the soil respiratory components' contribution (autotrophic respiration, $R_a$; heterotrophic respiration, $R_h$; hyphal respiration, $R_m$, and root respiration, $R_r$) to the total soil respiration at BIFoR FACE during the first two years of $CO_2$ enrichment. To our knowledge, this is the first study attempting to quantify the soil respiratory components in a mature temperate woodland under elevated $CO_2$ ($eCO_2$) using high-frequency automated measurements.

## 5.3 Methodology

A detailed description of the experimental site, Birmingham's Institute of Forest Research Free-Air Carbon Dioxide Enrichment (BIFoR FACE) facility, environmental drivers (VWC, $T_s$, and Pr) soil respiration components can be found in Chapter 2 of the thesis. This Chapter focuses on data collected during the Pre-treatment period (October 2016 –March 2017), Year 1, and Year 2 of $eCO_2$ enrichment (April 2017 – October 2018).

Briefly, BIFoR FACE is a temperate deciduous old-growth (>160 years) oak woodland located within Mill Haft at Staffordshire, England. The soil is brown earth cambisol with a sandy-clay texture and a mean soil pH of 4.5 at the top 10 cm. Mean soil total N and P in the top 10 cm are 0.28% and 18 mg P $kg^{-1}$, respectively, indicating that BIFoR is a low nutrient woodland, and total organic matter content is 3.67%.

A detailed FACE facility description can be found in Hart *et al.* (2019). Briefly, the BIFoR FACE facility comprises six 30 m diameter infrastructure arrays (elevated $CO_2$ (*e$CO_2$* hereafter), n = 3; controls (*Ambient*, hereafter), n = 3) and three non-infrastructure arrays. Year 1 of e$CO_2$ enrichment began on 3 April 2017 until 27 October 2017, while Year 2 began on the 26 March 2018 until 31 October 2018. e$CO_2$ enrichment took place from budburst to leaf fall approximately, and only during light hours, depending on the solar angle. During the first year of e$CO_2$ enrichment, e$CO_2$ arrays were within the 10% of target for 81.6% of the scheduled operation time and 20% of the target within 96.7% of scheduled operation time (Hart *et al.*, 2019). Contamination of the ambient arrays by $CO_2$ from the e$CO_2$ arrays was rare and short-lived, with ambient arrays being 10% of the control setpoint 98.8% of the time.

### 5.3.1 Measurements - Soil respiration and its partition components

Eighteen (18) experimental blocks were established across the site, 9 in ambient arrays and 9 in e$CO_2$ arrays (Figure 5.1), as proposed by Heinemeyer *et al.* (2007). Collars were installed in June 2016 (9 months before $CO_2$ fertilization), minimizing the effect of soil disturbance on soil respiration measurements, and left during the summer period for settlement; the soil collars were permanently installed to reduce soil

disturbance by repeated measurements and to ensure that the same soil was measured over time (King *et al.*, 2004).

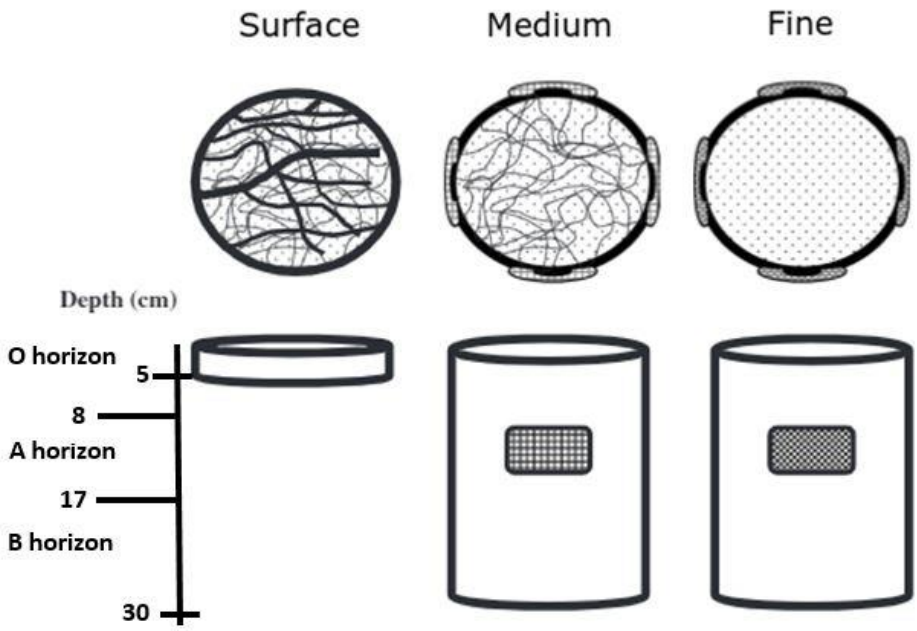


***Figure 5- 1:*** *Schematic representation of the soil respiration experimental blocks used at BIFoR FACE (adapted from Heinemeyer et al., 2007). Surface collars were only 5 cm deep inserted in the soil allowing roots, hyphal, and microbial growth. Medium and fine collars were inserted 30 cm deep in the soil, excluding fine roots from entering inside the collar. The fine collars were allowing only microbial growth.*

Each block consisted of one PVC shallow collar (Drainage Superstore, Plymouth, UK) and two deeper collars with different mesh treatments. All collars were 200 mm in diameter, the shallow collars (*surface* collars hereafter) were 5 cm in height, and the deeper collars were 30 cm in height. The shallow collar was inserted only 3 cm in the soil, pressed firmly onto the litter layer with three 20cm long stainless steel rods attached at the bottom of the collar to prevent dislocation by human or animal activity. The stainless steel rods ensured that no roots were severed while an airtight seal was achieved, avoiding potential lateral diffusion of $CO_2$. These collars did not interfere

with fine roots, mycorrhizal, or microbial dynamics and growth. The two deeper collars were inserted at approximately 25 cm depth, preventing fine root ingrowth which is concentrated mainly in the upper centimetres of the soil (Jackson, Mooney, and Schulze, 1997), and had 4 rectangular (5cm x 5 cm) windows cut into their sides which were covered with nylon meshes, with grids size of 41 µm (Ek, 1997) (*medium* collars hereafter) and 1 µm (*fine* collars hereafter) allowing or excluding ectomycorrhizal hyphal ingrowth, respectively. In summary, there are 3 surface, 3 medium, and 3 fine collars in every ambient and $eCO_2$ array (18 collars of each treatment across the site).

The experimental design suffered from new fine roots' growth inside the root exclusion collars (see section *5.8 Supplementary Material*). Higher respiration rates were observed in root exclusion collars than the associated surface collars. At the end of Year 2, the exclusion collars with such behaviours were excavated carefully without causing disturbance to the neighbouring collars of the block. All the exclusion collars found to be compromised by fine root ingrowth were excluded from the analysis, leading to three medium and ten fine collars excluded from the data analyses. In total, eighteen (9 in ambient and 9 in $eCO_2$ arrays) surface, 15 (8 ambient and 7 in $eCO_2$ arrays) medium, and 8 (4 in ambient and 4 in $eCO_2$ arrays) fine collars were used for the data analysis.

With the experimental design as described above, partitioning the soil respiration into total ($R_s$), autotrophic ($R_a$), and heterotrophic ($R_h$) was enabled. The $R_a$ was partitioned further to fine root ($R_r$) and hyphal ($R_m$) respiration. $R_s$ and $R_h$ were measured directly from the surface and fine collars, respectively. $R_a$ was calculated by subtracting the values of the fine collars from those of the surface collars, $R_r$ was calculated by subtracting the values of the medium collars from the surface collars, and lastly, $R_m$ was calculated by subtracting the values of the fine collars from the medium collars.

To enable a better understanding, please refer to *Chapter 2* section *2.2.1.1. Experimental design* where the equations used for the soil respiration partitioning calculations can be found.

Automated measurements of $R_S$ were taken at 1-hour intervals at all three experimental blocks in an $eCO_2$ array and its paired ambient array simultaneously, using 20-cm-diameter long-term chambers interfaced with a multiplexer and an infra-red gas analyser (IRGA) (Li-8100-104 long-term chambers, Li-8150 multiplexer, and Li-8100A IRGA; Licor). The observation length was 2 min with a 20 s deadband, a 15 s pre-purge, and a 45 s post purge, giving a total measurement cycle of 3 min 20 s per collar. The measurements began on 19 October 2016 (5 months before the beginning of $eCO_2$ enrichment), and we report data until 31 October 2018 (end of Year 2 of $eCO_2$ enrichment). The LI-COR systems were moved every two weeks between replicating paired eCO2 and ambient array and returning to the same array pair on a 4-week rotation.

All data went through quality checks before analysis using SoilFluxPro software, version 4.0.1 (LI-COR, Nebraska, USA). Data points with negative linear fluxes or data points with a linear flux coefficient of variation higher than 3.5 were removed from the dataset, as these were indicative of leaks in the chamber. Data points with a linear flux coefficient of variation between 1.5 and 3.5 were manually checked for assessing the fit quality. Of a total of 320,976 data points during the total period of operation as mentioned above, 195,124 data points were available for analysis, primarily due to equipment failures (water condensation in the electric plates) and, to a less extent, due to poor-quality fits. However, due to other experimental design failures (fine roots penetrated the root exclusion collars from the bottom and/or through the failed meshed windows), only 144,294 data points were used for this Chapter's analysis. That led to

an approximately 50% data loss in total. Daily and monthly averages of these data are reported in this Chapter in μmol $CO_2$ $m^{-2}$ $s^{-1}$.

### 5.3.2 Statistical analysis

All analyses were carried out using R 3.6.2 and Rstudio 1.2.5033 (RStudio Team, 2019). However, due to the limitations of the experimental design's failure, only descriptive statistics were performed for this Chapter.

## 5.4 Results

Fig.5-2 shows Rh, Ra, Rr, and Rm's seasonal course during the first two years of $eCO_2$ enrichment at BIFoR FACE. During Year 1, all soil respiratory components exhibited the same seasonal pattern, increasing until mid-summer, followed by a decrease as the growing season came to an end. Both $R_a$ and $R_h$ were higher in $eCO_2$ arrays during the first year of $eCO_2$ enrichment. Subsequently, $R_a$ was further partitioned to $R_r$ and $R_m$. Although $R_m$ was observed to respond to $eCO_2$ as the $R_h$, $R_r$ was the only soil respiratory component to exhibit higher rates in ambient arrays.

All soil respiratory components exhibited low rates during the Dormant period. During Year 2, $R_h$ had the same response towards $eCO_2$ as in Year 1 (higher in $eCO_2$ arrays). However, the seasonal pattern was not the same. $R_h$ in ambient arrays exhibited the lowest rates in late spring (May 2018) and gradually increased as the growing season finished. Contrary, $R_h$ in $eCO_2$ arrays increased until early summer, followed by a decrease in July 2018, only to increase again. $R_a$ was observed to follow a similar seasonal pattern as $R_h$; higher rates at the beginning of the growing season, followed by a decrease in midsummer rates. Due to the experimental design limitations and

instrumental failures, no clear conclusions can be drawn once $R_a$ was further partitioned to $R_r$ and $R_m$.

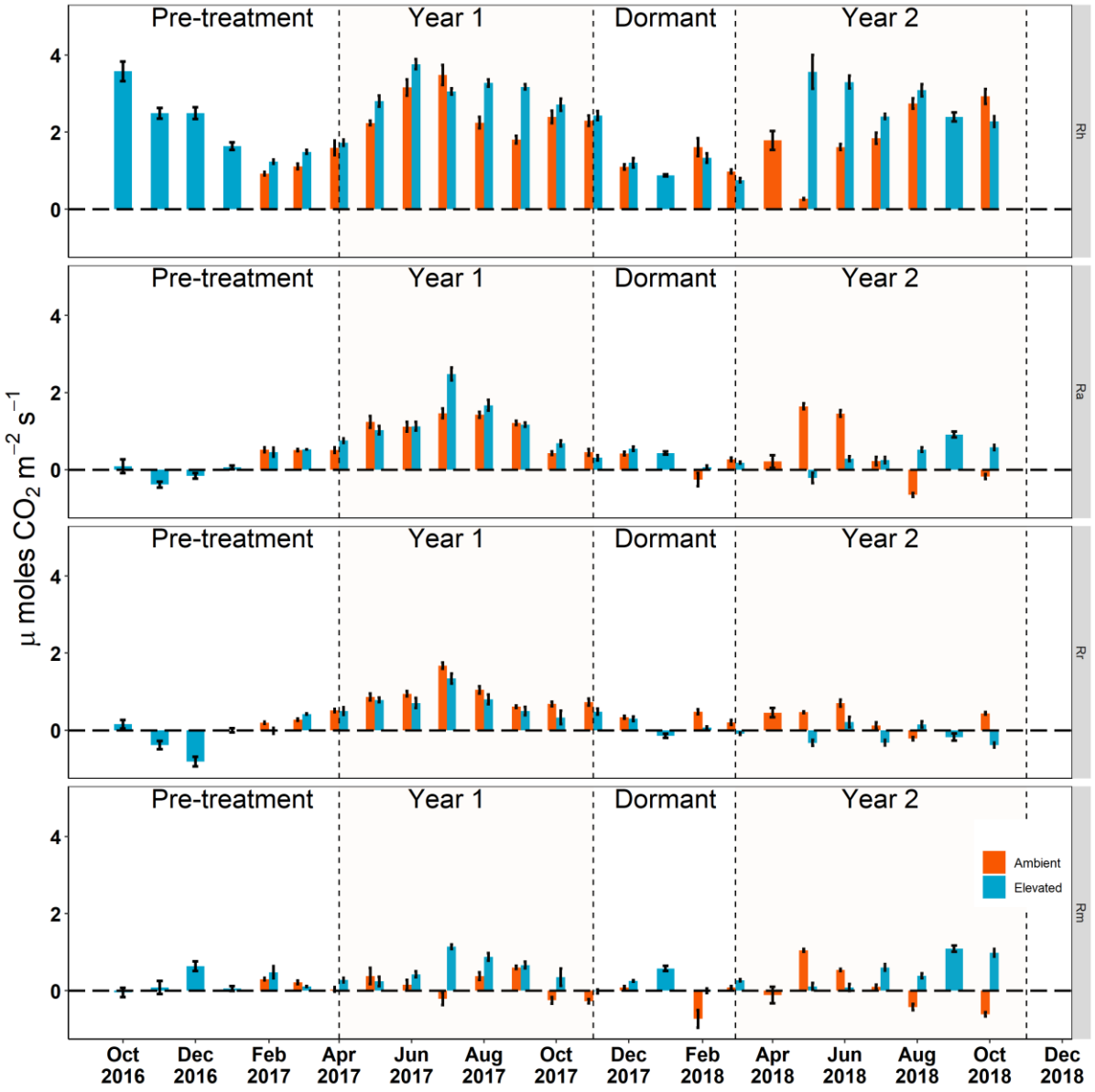


***Figure 5- 2:*** *Seasonal course of heterotrophic respiration, $R_h$; autotrophic respiration $R_a$; root respiration, $R_r$, and hyphal respiration, $R_m$ during the Pre-treatment and first two years of $eCO_2$ enrichment (October 2016 – October 2018). The blue coloured bars denote the $eCO_2$ arrays, while the orange bars denote the ambient arrays. All data describe monthly averages, while the error bars denote standard error*.

Figure 5-3 shows the percentage contribution of all soil respiratory components ($R_h$, $R_a$, $R_r$, and $R_m$) to total soil respiration, on an annual basis, in ambient and $eCO_2$ arrays

for the first two years of $eCO_2$ enrichment at BIFoR FACE. $R_h$ was observed to be the main contributor of soil respiration, responsible for over 70 and 80% respiration rates during Year 1 and 2 of $eCO_2$ enrichment, respectively. By further partitioning $R_a$, $R_r$ was the main contributor of $R_a$, accounting for over half of the $R_a$ rates during Year 1. For Year 2, $R_a$ partition was limited by experimental design and instrumental failures.

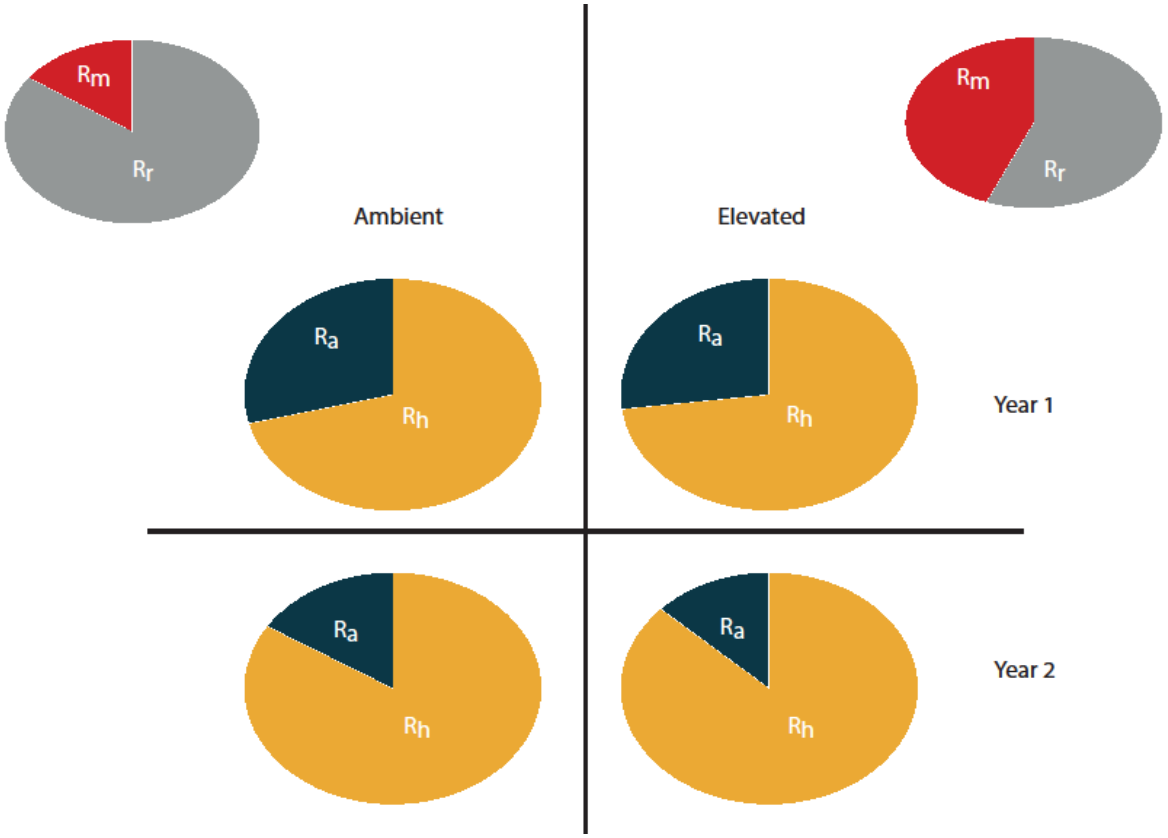


***Figure 5- 3:*** *Percentage contribution of soil respiratory components ($R_h$, $R_a$ $R_r$, and $R_m$), on an annual basis, to total soil respiration in ambient and $eCO_2$ arrays during the first two years of $eCO_2$ enrichment at BIFoR FACE. Note that partitioning $R_a$ to $R_r$ and $R_m$ during Year 2 was not possible.*

$R_h$ and $R_a$ contribution in ambient and $eCO_2$ arrays were similar during Year 1, with $R_a$ contributing less in the total soil respiration in $eCO_2$ than in ambient arrays ($R_h$: 71% in ambient vs. 73% in $eCO_2$ arrays; $R_a$: 29% in ambient vs. 27% in $eCO_2$ arrays). Once $R_a$ was partitioned to $R_r$ and $R_m$, $R_r$ was responsible for 85% of the $R_a$ in ambient arrays ($R_r$ 85% and $R_m$ 15% in ambient arrays). Interestingly, in $eCO_2$ arrays, although

$R_r$ was found to be the main $R_a$ contributor, $R_m$ contribution was greater than the one in ambient arrays, responsible for almost half of the $R_a$ ($R_r$ 56% and $R_m$ 44% in $eCO_2$ arrays). In Year 2, although $R_h$ was again the main contributor of soil respiration, the contribution was higher than Year 1 (84% vs. 87% in ambient and $eCO_2$ arrays, respectively), forcing $R_a$ to contribute less (16% vs. 13% in ambient and $eCO_2$ arrays, respectively).

All respiratory components were observed to be higher in $eCO_2$ arrays during the first year of the $eCO_2$ enrichment, except for $R_r$, which was higher in ambient arrays (Fig. 5-4). Specifically, $R_h$, $R_a$, and $R_m$ were higher by 17, 7, and 217%, respectively, in $eCO_2$ arrays, while $R_r$ was 40% higher in ambient arrays. During the second year of $eCO_2$ enrichment, $R_h$ was 29% higher in $eCO_2$ than in ambient arrays, whereas $R_a$ was similar between ambient and $eCO_2$ arrays (only 0.8% higher in ambient arrays).

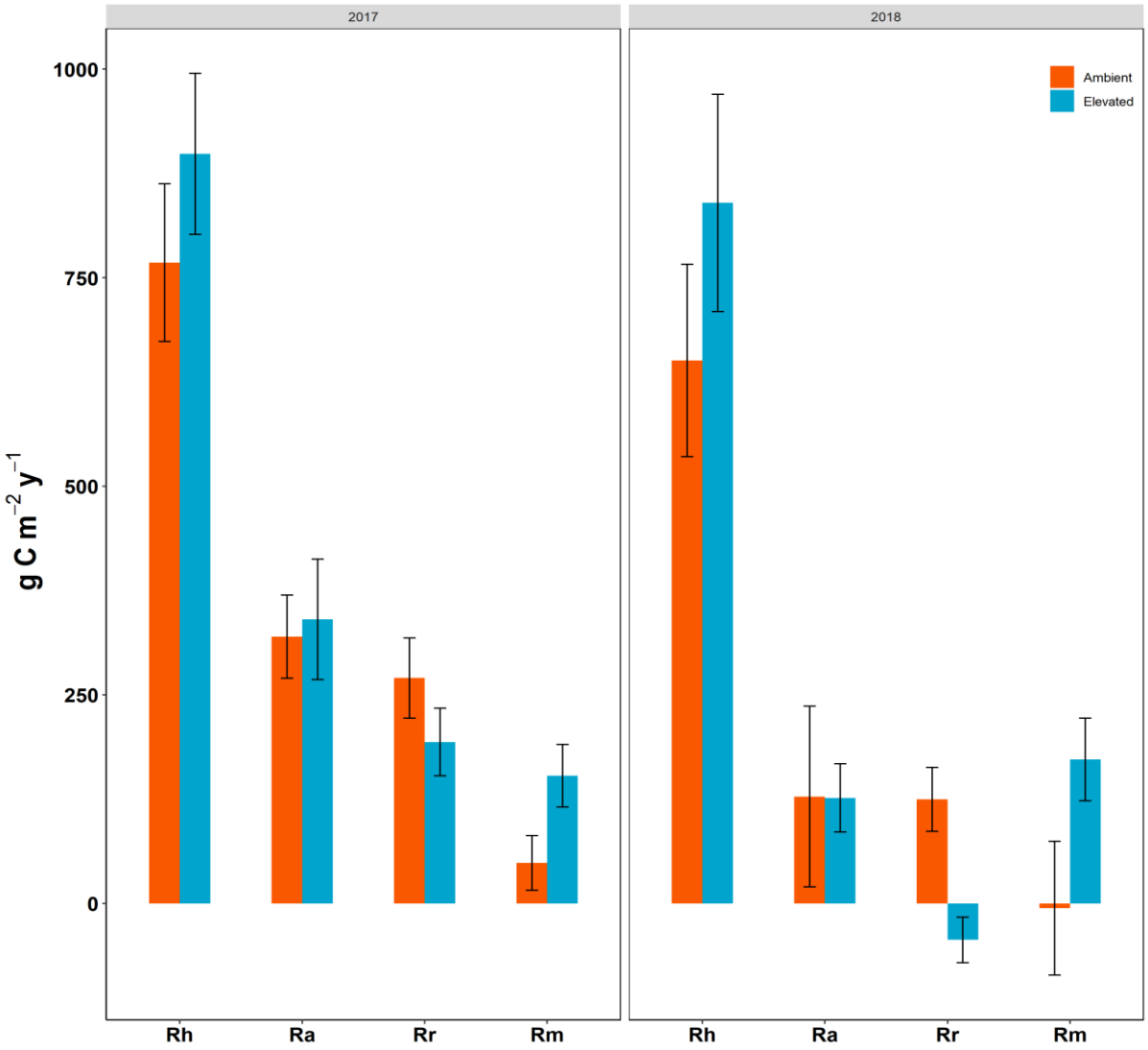


***Figure 5- 4:*** *Mean annual $R_h$, $R_a$, $R_r$, and $R_m$ in ambient (orange) and $eCO_2$ (blue) arrays during the first two years of $eCO_2$ enrichment at BIFoR FACE. The error bars denote the se.*

In 2017 (Year 1) all soil respiratory components had very weak negative responses on VWC (Fig. 5.5). The relationship between $R_h$ and VWC was marginally significant in both ambient and $eCO_2$ arrays, while the relationship between both $R_a$ and $R_r$ and VWC in ambient arrays was significant. In contrast, in $eCO_2$ arrays, the relationships between $R_a$ and $R_r$ and VWC were marginally significant and not significant, respectively. $R_m$ relationship with VWC was significant only in $eCO_2$ arrays, while in ambient arrays, the relationship was observed not to be significant. All respiratory

components were more sensitive to VWC in ambient arrays, except for $R_m$, which was more sensitive to VWC in $eCO_2$ arrays (see section *5.8 Supplementary Material*, Table S5-1).

During Year 2 (2018), the soil respiratory components' responses on VWC were different from those in Year 1 (Fig. 5-5). All respiratory components were more sensitive to VWC in $eCO_2$ arrays (see section *5.8 Supplementary Material*, Table S5-1). Moreover, all respiratory components ($R_a$, $R_r$, and $R_m$) in ambient arrays had a very weak positive response on VWC, except $R_h$, which had a weak negative response on VWC. The soil respiratory components' responses on VWC in $eCO_2$ arrays were precisely the opposite of those observed in ambient arrays. All soil respiratory components ($R_a$, $R_r$, and $R_m$) had a weakly positive response on VWC, except $R_h$, which had a moderate response on VWC. All correlations were significant, except $R_a$, $R_r$, and $R_m$ and VWC in ambient arrays that were not significant, and Rr and VWC in eCO2 arrays were marginally significant.

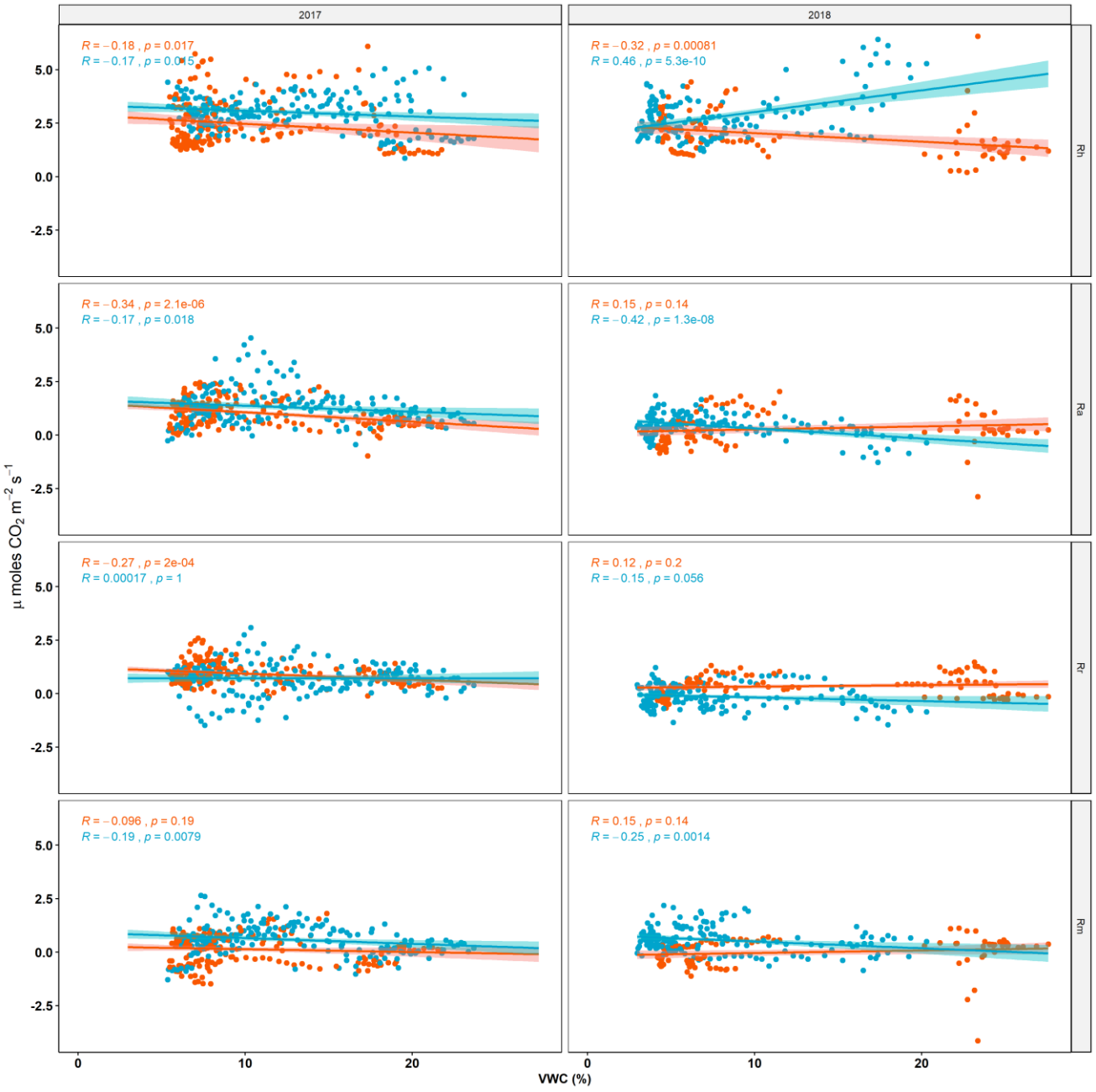


***Figure 5- 5:*** *$R_h$, $R_a$, $R_r$, and $R_m$ responses on VWC in both ambient and $eCO_2$ arrays during the first two years of $eCO_2$ enrichment at BIFoR FACE. The blue solid points represent the $eCO_2$ arrays, while the orange solid points represent the ambient arrays.*

Both $R_h$ and $R_a$ respiratory components were observed to have moderate but significant relationships with $T_s$ during the first year of $eCO_2$ enrichment (Fig. 5-6). The relationship was stronger under $eCO_2$ for $R_h$, while T and Ra's relationship was similar between ambient and $eCO_2$ arrays. $R_r$ and soil temperature relationship were moderate and weak in ambient and $eCO_2$ arrays, respectively, but significant.

However, $R_m$ was observed to have precisely the opposite pattern than $R_r$; $R_m$ and T relationship was weak and not significant in ambient arrays. The relationship was moderate but significant in $eCO_2$ arrays. $R_h$ and $R_r$ were more sensitive to $T_s$ in ambient arrays, whereas $R_a$ and $R_m$ were more sensitive to $T_s$ in $eCO_2$ arrays (see section *5.8 Supplementary Material*, Table S5-1).

However, during the second year of $eCO_2$ enrichment, the relationship between $T_s$ and all respiratory components changed either weak or no relationship at all (Fig. 5-6). Both $R_a$ and $R_h$ components had a weak and non-significant relationship with T in ambient arrays. In $eCO_2$ arrays, the $R_h$ and $R_a$ relationships with $T_s$ were weak positive and negative, respectively, albeit both significant. The further $R_a$ partitioning, $R_r$ and $R_m$, had no relationship with T in ambient arrays. In $eCO_2$ arrays, the $R_r$ relationship with $T_s$ was weak and not significant, while $R_m$ had a moderate relationship with $T_s$, but it was significant. All respiratory components were more sensitive to T in $eCO_2$ arrays during the second year of the experiment (see section *5.8 Supplementary Material*, Table S5-1).

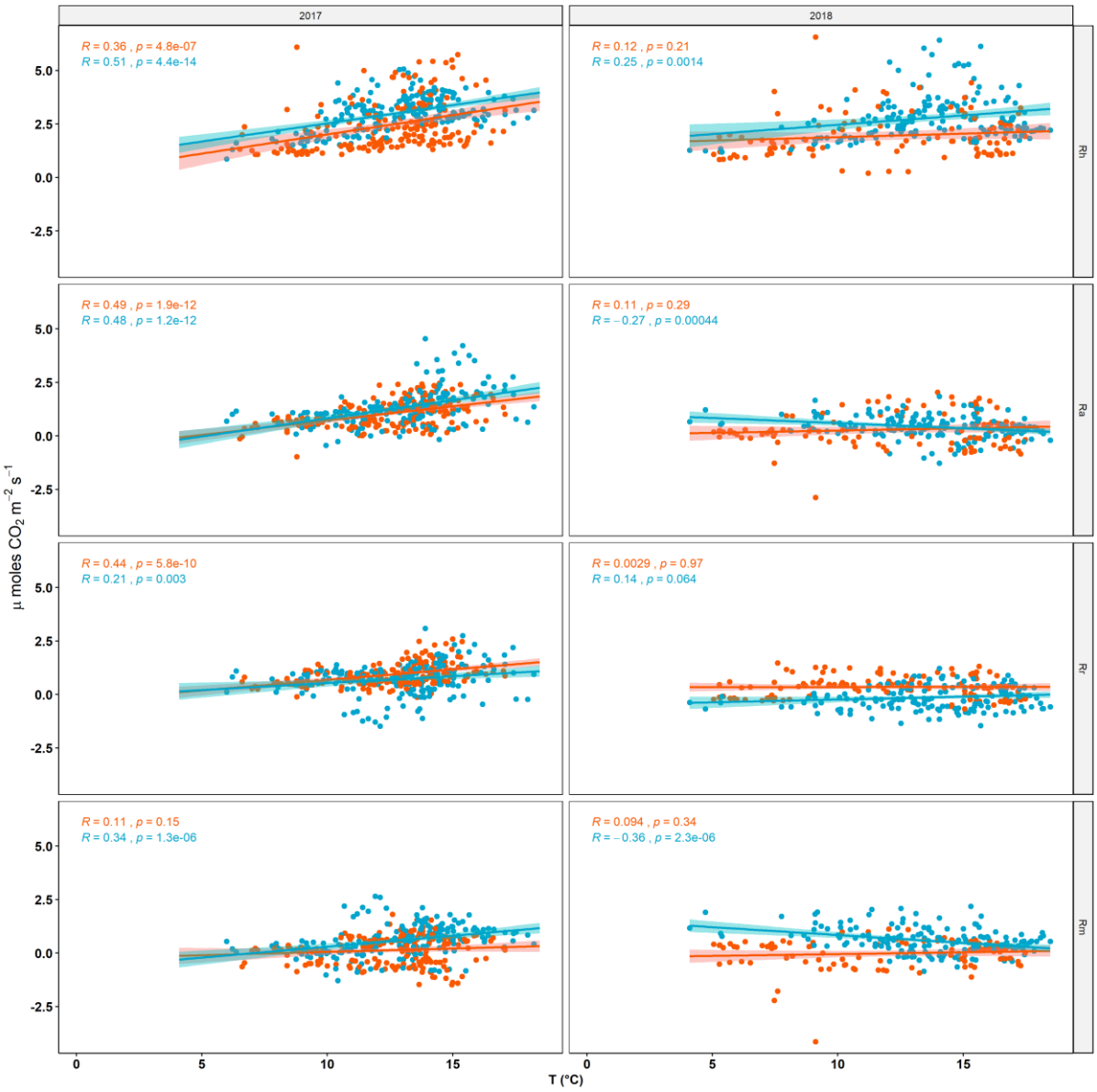


**Figure 5- 6:** *$R_h$, $R_a$, $R_r$, and $R_m$ responses on $T_s$ in both ambient and $eCO_2$ arrays during the first two years of $eCO_2$ enrichment at BIFoR FACE. The blue solid points represent the $eCO_2$ arrays, while the orange solid points represent the ambient arrays.*

## 5.5 Discussion

One of the most popular techniques for partitioning soil respiration into components, especially in forest ecosystems, is the root exclusion/ trenching technique (Bowden *et al.*, 1993; Buchmann, 2000; Ross *et al.*, 2001; Lee *et al.*, 2003; Heinemeyer *et al.*, 2007). It is a simple technique with a relatively easy and cheap installation that could be appropriate for long-term measurements, ensuring repeatability. In principle, the

technique should provide a robust estimate of the root-, hyphal- and heterotrophic-derived $CO_2$. In reality, though, the exclusion technique can only provide a rough estimate of the different soil components'-derived $CO_2$ due to multiple limitations observed between root inclusion and root exclusion soil, such as a) different microclimatic conditions (Fisher and Gosz, 1986; Ross *et al.*, 2001), b) altered nutrient cycles (Dormaar, 1990; Kuzyakov, 2002; Paterson *et al.*, 2003; Cheng and Kuzyakov, 2005), and c) experimental design failures (section *5.8 Supplementary material* Fig. S5-7). Due to the above limitations and the other instrumental failures experienced in the two years, caution must be taken when extrapolating the results of this chapter, especially during Year 2, when the autotrophic respiration component partitioning became impossible.

This Chapter assessed soil respiration's partition to its respiratory components (heterotrophic, autotrophic, root, and mycorrhizal respiration) during the first two years of $eCO_2$ enrichment at BIFoR FACE. Each component was observed to have strong seasonal patterns during both years of $eCO_2$ enrichment (Fig. 5-2). All soil respiratory components were found to have higher fluxes during the growing seasons and lower fluxes during dormant seasons. The soil respiratory components' seasonal patterns observed in this Chapter follow similar seasonal patterns with the abiotic factors (soil moisture and soil temperature) described in *Chapters 3* and *4,* in which fluxes increased as soil temperature increased and soil moisture decreased.

Soil moisture was observed to have significant but weak effects on both autotrophic and heterotrophic respiratory components (Fig. 5-5) in both ambient and $eCO_2$ arrays during Year 1 of $eCO_2$ enrichment at BIFoR FACE. Exceptions on these observations were the root respiration in $eCO_2$ arrays and mycorrhizal respiration in ambient arrays, where soil moisture was observed not to affect them. Both heterotrophic and

autotrophic respiratory components were observed to have moderate but significant relationships with soil temperature during the first year of $eCO_2$ enrichment (Fig. 5-6), while the relationships between soil temperature and the autotrophic partition components (root and mycorrhizal components) were observed to be weaker but still significant (except $R_m$ in ambient arrays in which the relationship was not significant).

The relationship patterns between soil moisture and soil respiratory components during Year 2 differ substantially from those observed during Year 1 (Fig. 5-5). Only the heterotrophic component had a moderate negative relationship with soil moisture, which was significant. In contrast, the autotrophic component, including root and mycorrhizal components, had very weak positive relationships with soil moisture that was not significant. In $eCO_2$ arrays, the exact opposite pattern was observed. Only the heterotrophic component had a moderately positive relationship with soil moisture. In contrast, the autotrophic components, including root and mycorrhizal respirations, had moderate and weak negative relationships with soil moisture, respectively. All relationships between soil moisture and soil respiratory components in $eCO_2$ arrays were significant during Year 2 of $eCO_2$ enrichment at BIFoR FACE.

During the second year of $eCO_2$ enrichment (extreme events year; see *Chapter 4*), all soil respiratory components had no relationship with soil temperature in ambient arrays. In $eCO_2$ arrays, only the heterotrophic respiration was observed to correlate with soil temperature positively; weak but significant. Soil temperature was observed to have a significant but weak negative effect on autotrophic respiration, and the same effect was observed with mycorrhizal respiration. Soil temperature did not affect root respiration in $eCO_2$ arrays.

During the first year of $eCO_2$ enrichment at BIFoR FACE, the heterotrophic and autotrophic component of soil respiration responded similarly to abiotic factors (soil moisture and soil temperature; Fig. 5-5 and 5-6 respectively), although it has been observed that both components can respond differently to biotic and abiotic controlling factors, as well as their interactions (Trueman and Gonzalez-Meler, 2005; Kuzyakov and Gavrichkova, 2010; Taneva and Gonzalez-Meler, 2011). The heterotrophic component was slightly less sensitive to soil moisture than the autotrophic component in both ambient and $eCO_2$ arrays during Year 1 (Gomez-Casanovas *et al.*, 2012).

Moreover, the heterotrophic component was more sensitive to soil temperature than the autotrophic component in ambient arrays (Gomez-Casanovas *et al.*, 2012), while they had the same sensitivity in $eCO_2$ arrays. During the second year of $eCO_2$ enrichment, the heterotrophic component was more sensitive to both soil moisture and temperature in both ambient and $eCO_2$ arrays. Interestingly our data are in agreement with studies in grasslands and prairies (Luo *et al.*, 2001; Hartley *et al.*, 2007; Bahn *et al.*, 2009), microcosms model systems (Bååth and Wallander, 2003), and forest ecosystems (Heinemeyer *et al.*, 2007; Högberg, 2010; Wei, Weile and Shaopeng, 2010).

The extreme events stressors altered the sensitivities of the soil respiratory components to abiotic factors. In ambient arrays, all respiratory components were less sensitive to soil moisture in Year 2 versus Year 1, except for the heterotrophic component, which had the same sensitivity to soil moisture both years. However, in $eCO_2$ arrays, all soil respiratory components were more sensitive to soil moisture in Year 2. Moreover, both autotrophic and heterotrophic components were less sensitive to soil temperature in both ambient and $eCO_2$ arrays in Year 2.

Although the general patterns in the relationships between the soil respiratory components and the abiotic factors were similar during the first year of $eCO_2$ enrichment, their sensitivities to the abiotic factors differed between ambient and $eCO_2$ arrays. Soil respiratory components were less sensitive to soil moisture in $eCO_2$ arrays, except for mycorrhizal respiration, which was less sensitive to soil moisture in ambient arrays (section *5.8 Supplementary Material*, Table S5-1). Moreover, only the autotrophic and mycorrhizal respiration were more sensitive to soil temperature in $eCO_2$ arrays. During the second year of the experiment, which concurred with a series of extreme events, all soil respiratory components were more sensitive to both soil moisture and soil temperature in $eCO_2$ arrays.

Heterotrophic respiration was observed to be the main contributor to soil respiration in both years, accounting for almost ¾ of the total soil respiration in both ambient and $eCO_2$ arrays (Fig. 5-3). Our data agree with previous studies conducted in temperate forests (Matamala and Schlesinger, 2000; Subke, et al., 2006; Taneva and Gonzalez-Meler, 2011). By further attempting to partition autotrophic respiration to root and mycorrhizal respiration (only possible for the first year of $eCO_2$ enrichment), root respiration was the main contributor of autotrophic respiration in both ambient and $eCO_2$ conditions (autotrophic partitioning data available only for Year 1). Although the autotrophic/heterotrophic contribution ratio was similar between ambient and $eCO_2$ arrays during Year 1 of $eCO_2$ enrichment, the root and mycorrhizal contribution was substantially altered in $eCO_2$ arrays, with mycorrhizal respiration contributing 29% more to autotrophic respiration. This observation agrees with previous studies (Staddon, Gregersen and Jakobsen, 2004), where mycorrhizal abundance was observed to be stimulated under $eCO_2$ by increased C allocation belowground.

Heterotrophic respiration was observed to contribute more to total soil respiration during Year 2 in ambient and $eCO_2$ arrays. Autotrophic respiration contributed less to total soil respiration during the second year of the experiment. During the second year of $eCO_2$ enrichment, UK experienced a prolonged heatwave during the summer season (see *Chapter 4*). Our data agree with other studies conducting warming or drought experiments (Schimel, Balser and Wallenstein, 2007; Manzoni, Schimel and Porporato, 2012; Deslippe *et al.*, 2016; Sun, Lei and Chang, 2019). The heterotrophic component was observed to be less sensitive than the autotrophic component to drought-induced stress. Moreover, before the heatwave, the woodland suffered from a winter-moth infestation (see Chapter 4). Insect infestations have been observed to alter the forest floor microclimate by altering leaf litter quantity and chemical quantity (Grace, 1986; McInnes *et al.*, 1992; Findlay *et al.*, 1996; Grime *et al.*, 1996; Chapman *et al.*, 2003), deposition of insect by-products (Hollinger, 1986; Frank and Groffman, 1998; Christenson *et al.*, 2002).

Autotrophic respiration was almost halved during the second year compared to the first year of $eCO_2$ enrichment at BIFoR FACE. The autotrophic component has been observed to contribute less to the total soil respiration under drought stress (Balogh *et al.*, 2016; Sun, Lei and Chang, 2019). Drought stress has been observed to affect root respiration by interfering with the root physiological processes (Linn and Doran, 1984; Zhang *et al.*, 2014) by reducing the supply of photosynthates and other residues to the root systems (Carbone *et al.*, 2011; Gomez-Casanovas *et al.*, 2012).

Although heterotrophic respiration was higher in $eCO_2$ arrays, on an annual basis, both years of $eCO_2$ enrichment, autotrophic respiration was higher in $eCO_2$ arrays only the first year of the experiment. In the second year of the experiment, autotrophic respiration was similar between ambient and $eCO_2$ arrays. The two autotrophic

components, roots, and mycorrhizae had an adverse response between ambient and $eCO_2$ arrays, with root respiration being the only soil respiratory component lower in $eCO_2$ arrays.

## 5.6 Conclusions

This chapter aimed to monitor the soil-derived $CO_2$ and attempt to quantify the soil respiratory components' contribution during the first two years of $eCO_2$ enrichment at BIFoR FACE. During the first year of the experiment, although the ecosystem was still in flux, $eCO_2$ stimulated the total soil respiration, and by partitioning the respiration to two primary sources (autotrophic and heterotrophic component), we observed that both components were releasing more $CO_2$ under $eCO_2$, with the heterotrophic component having higher fluxes. Once the autotrophic component was partitioned further to root and hyphal respiration, we found that root respiration was lower in $eCO_2$ arrays, while hyphal respiration was higher in $eCO_2$ arrays.

It is generally observed that $eCO_2$ stimulates root respiration (Schlesinger *et al.*, 2006); however, our results disagree with this observation. Lower root respiration in $eCO_2$ arrays combined with over 100% hyphal stimulation and a significant decrease in nitrate availability (Chapter 3) might indicate that trees were probably under a nutrient limitation stress; thus, they shifted their carbon allocation to hyphal structures to forage more nutrients. Mature forests have very tight nutrient cycles, and they rely almost entirely upon internal nutrient recycling to meet their nutrient demands. If such a pattern (decreased nutrient availability and higher hyphal fluxes), is not a short-term response, trees might not be able to uptake extra $CO_2$ because of nutrient limitations.

Moreover, the soil respiratory components had different sensitivities towards the abiotic factors (soil temperature and soil moisture), and $eCO_2$ was observed to alter those sensitivities. It is essential to highlight that the different respiratory components have different carbon turnover rates and mean residence time, with root respiration having the fastest turnover and shortest mean residence time. On the other side of the spectrum, basal respiration (microbial respiration due to soil organic matter decomposition) has the slowest turnover and longest mean residence time. Thus, shifts in the relationships between soil respiratory components and abiotic factors due to $eCO_2$ can greatly impact the C cycle and the ecosystems' potential of sequestering C in the long-term.

Future predictions give a warning for an increase in the frequency and intensity of extreme events. Prolonged heatwave combined with defoliation event shifted the relative contribution of autotrophic and heterotrophic components, with the heterotrophic almost entirely dominating the total respiration. The interaction of extreme events and $eCO_2$ intensified this observation. As mentioned before, due to the different turnover rates and mean residence time that soil respiratory components have, the C cycle might be severely affected since microbial respiration releases older soil C to the atmosphere and thus posing a threat to the ecosystem's capacity of acting as a sink. However, these observations were only short-term, and it is unclear whether there is the potential of heritage effects of the extreme events to the ecosystem in the long term or whether such events have a short lifetime in the ecosystem's cycling.

## 5.7 References

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## 5.8 Supplementary material

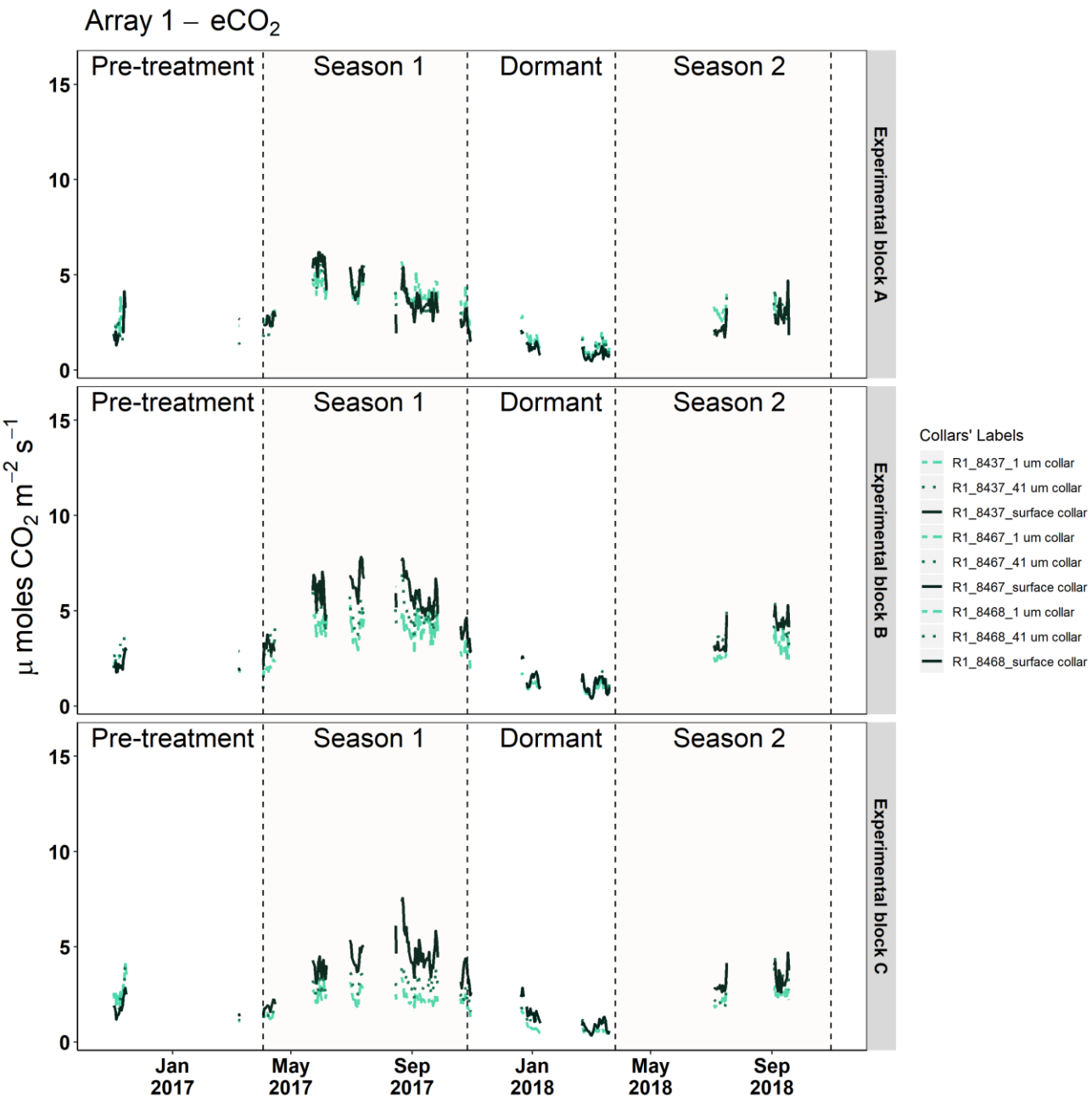


***Figure S5- 1:*** Investigation of experimental design failures for all three experimental blocks in Array 1 ($eCO_2$ array) in daily averages during the first two years of $eCO_2$ enrichment at BIFoR FACE. Surface collars are denoted with dark green solid lines, medium collars are denoted with green dotted lines, while fine collars are denoted with light green dashed lines.

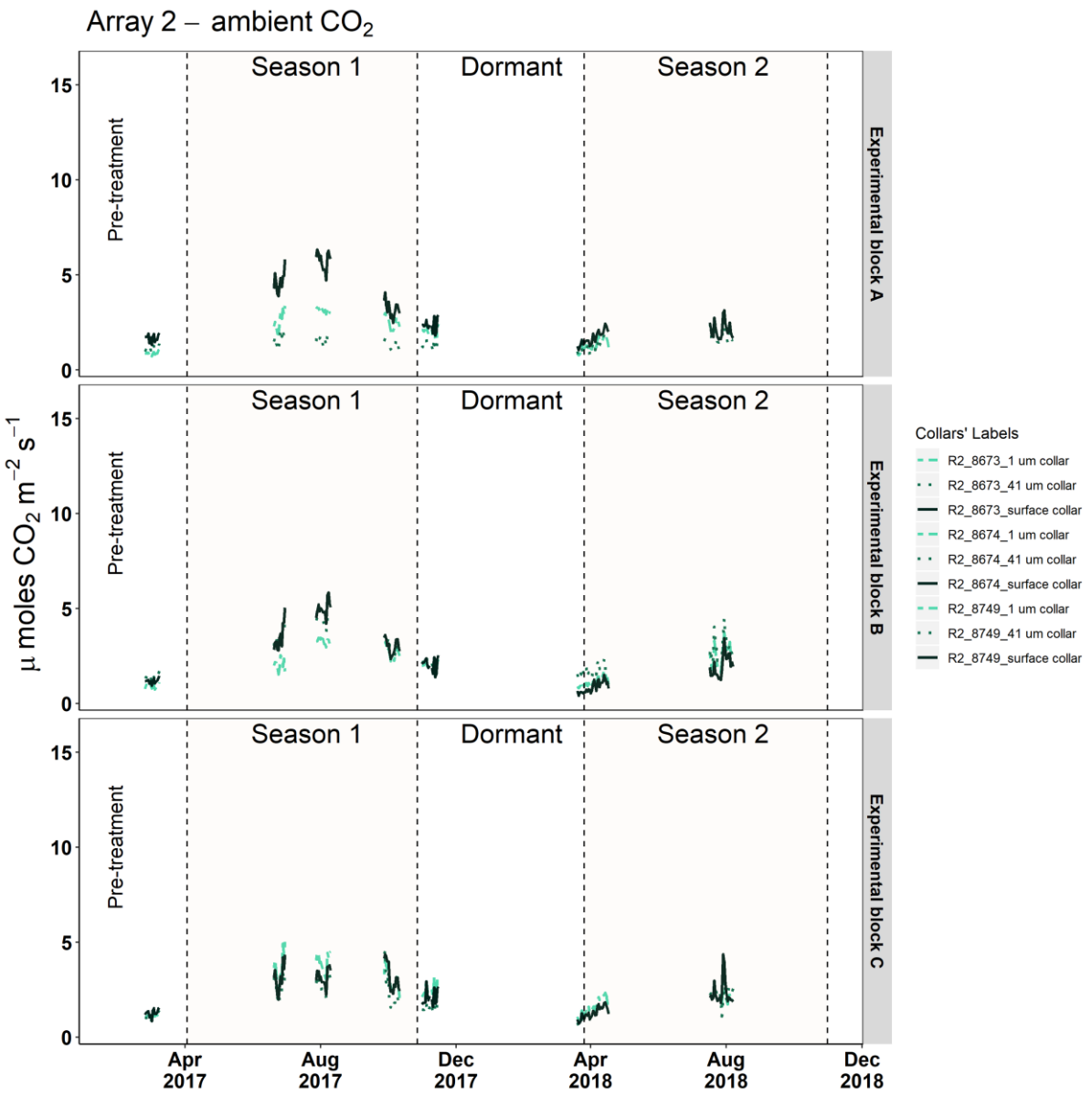


***Figure S5- 2:*** *Investigation of experimental design failures for all three experimental blocks in Array 2 (ambient array) in daily averages during the first two years of $eCO_2$ enrichment at BIFoR FACE. Surface collars are denoted with dark green solid lines, medium collars are denoted with green dotted lines, while fine collars are denoted with light green dashed lines.*

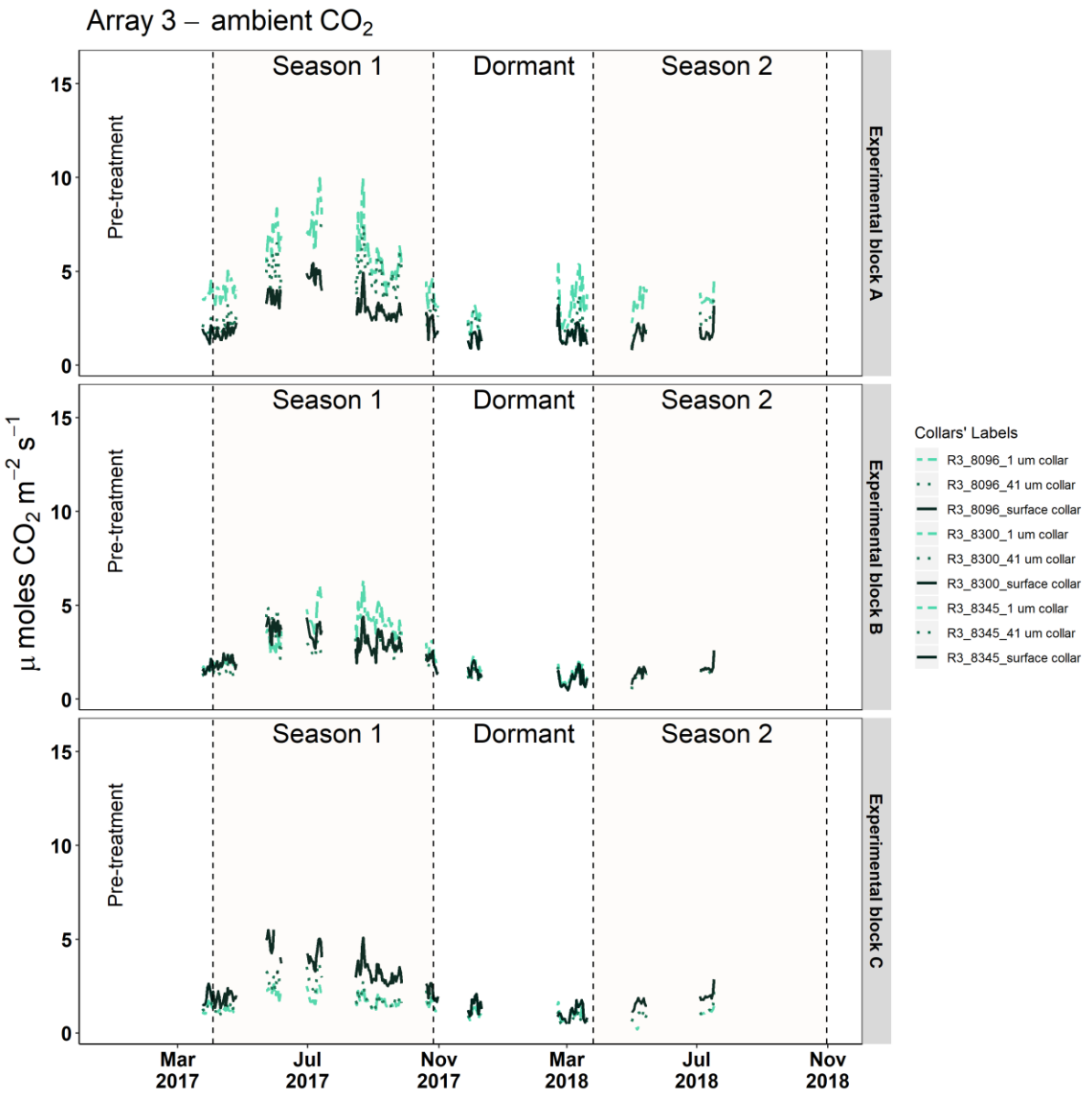


***Figure S5- 3:*** *Investigation of experimental design failures for all three experimental blocks in Array 3 (ambient array) in daily averages during the first two years of $eCO_2$ enrichment at BIFoR FACE. Surface collars are denoted with dark green solid lines, medium collars are denoted with green dotted lines, while fine collars are denoted with light green dashed lines.*

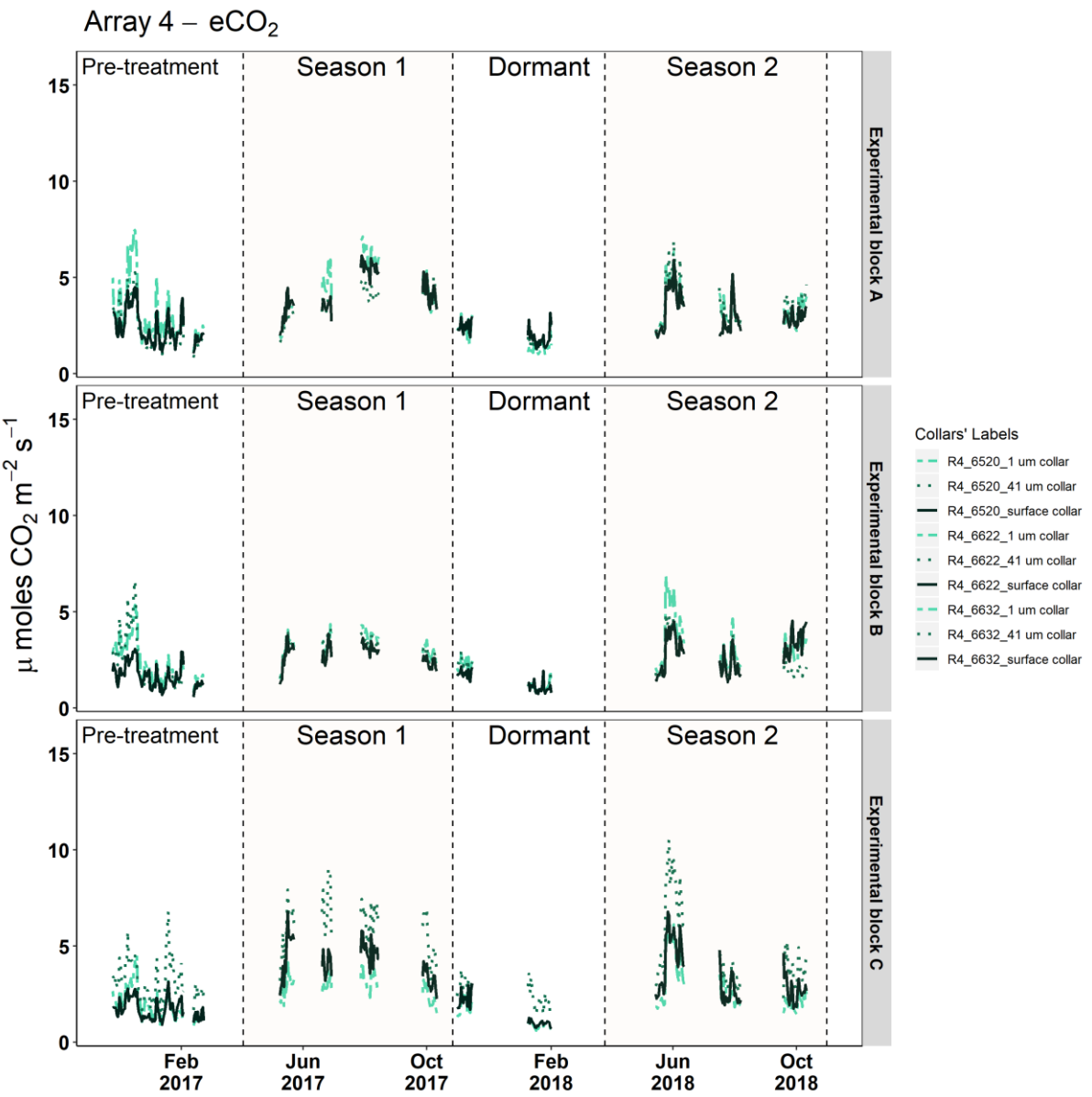


***Figure S5- 4:*** *Investigation of experimental design failures for all three experimental blocks in Array 4 ($eCO_2$ array) in daily averages during the first two years of $eCO_2$ enrichment at BIFoR FACE. Surface collars are denoted with dark green solid lines, medium collars are denoted with green dotted lines, while fine collars are denoted with light green dashed lines.*

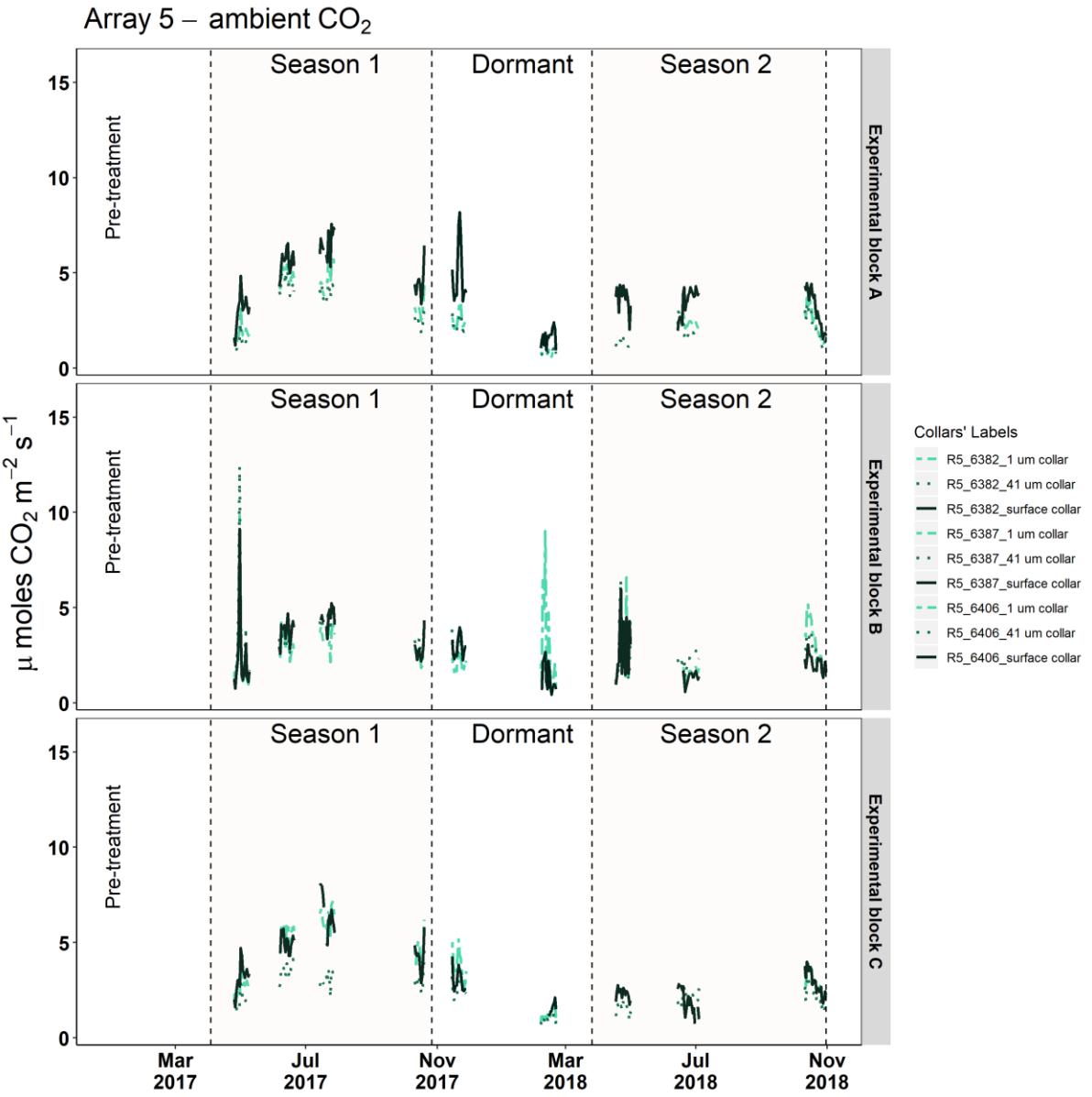


***Figure S5- 5:*** *Investigation of experimental design failures for all three experimental blocks in Array 5 (ambient array) in daily averages during the first two years of $eCO_2$ enrichment at BIFoR FACE. Surface collars are denoted with dark green solid lines, medium collars are denoted with green dotted lines, while fine collars are denoted with light green dashed lines.*

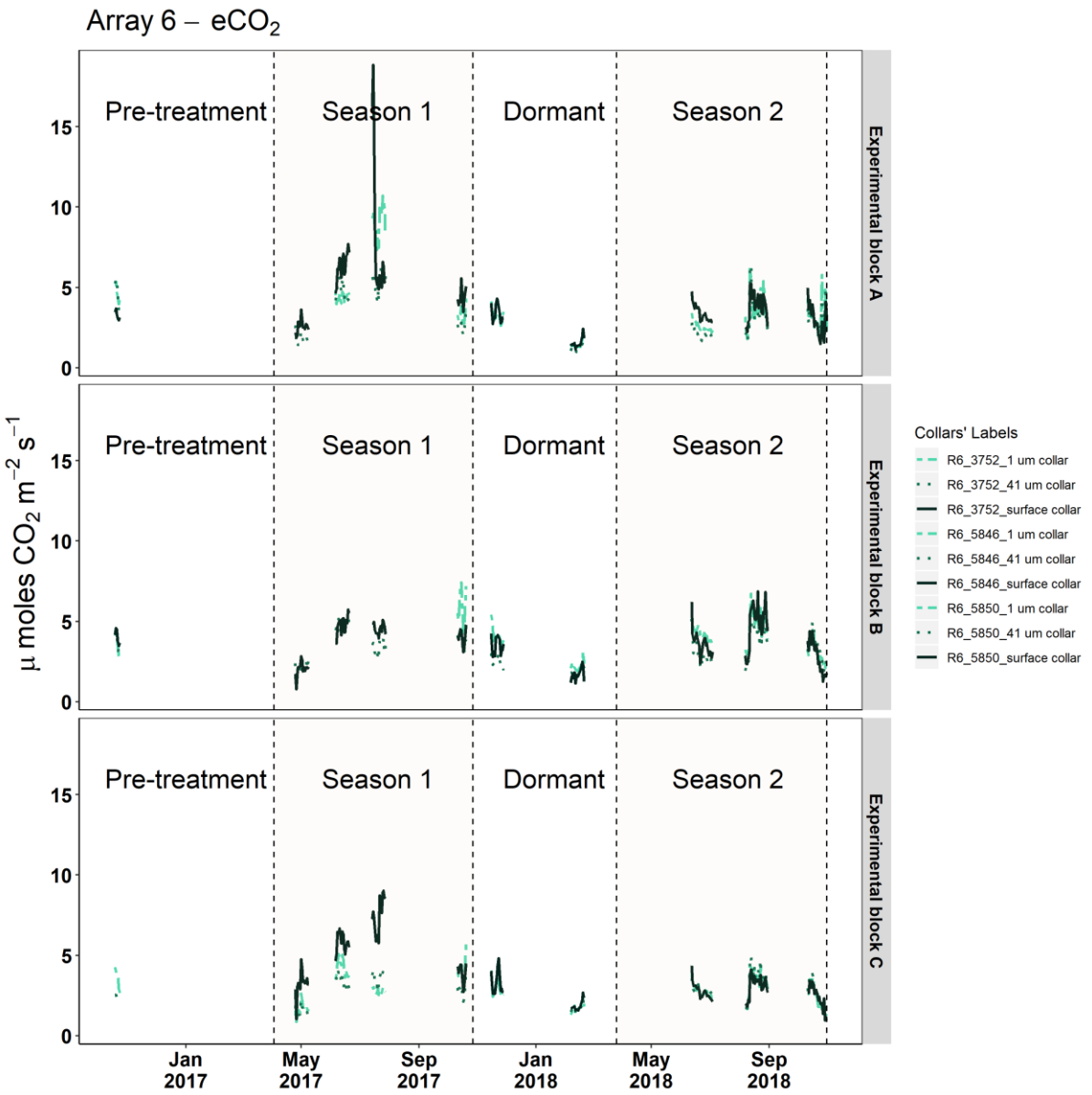


***Figure S5- 6:*** *Investigation of experimental design failures for all three experimental blocks in Array 6 ($eCO_2$ array) in daily averages during the first two years of $eCO_2$ enrichment at BIFoR FACE. Surface collars are denoted with dark green solid lines, medium collars are denoted with green dotted lines, while fine collars are denoted with light green dashed lines.*

***Table S5- 1:*** *Linear equations retrieved from VWC and T correlations with $R_h$, $R_a$, $R_r$ and $R_m$ (daily averages) during Year 1 and 2 at BIFoR FACE.*

| | Year 1 | | Year 2 | |
|---|---|---|---|---|
| | Ambient | $eCO_2$ | Ambient | $eCO_2$ |
| *VWC* | | | | |
| $R_h$ | y = 2.9 – 0.041x | y = 3.3 – 0.027x | y = 2.4 – 0.04x | y = 2 + 0.1x |
| $R_a$ | y = 1.5 – 0.044x | y = 2.7 – 0.028x | y = 0.13 + 0.014x | y = 0.79 – 0.047x |
| $R_r$ | y = 1.2 – 0.028x | y = 0.72 + 2.5* $10^{-5}$ x | y = 0.26 + 0.0068x | y = -0.0025 – 0.017x |
| $R_m$ | y = 0.27 – 0.013x | y = 0.92 – 0.027x | y = 0.16 + 0.012x | y = 0.82 – 0.032x |
| *T* | | | | |
| $R_h$ | y = 0.22 + 0.18x | y = 0.83 + 0.17x | y = 1.6 + 0.033x | y = 1.6 + 0.087x |
| $R_a$ | y = -0.61 + 0.13x | y = -0.87 + 0.17x | y = 0.037 + 0.021x | y = 1.1 - 0.048x |
| $R_r$ | y = -0.3 + 0.098x | y = -0.12 + 0.066x | y = 0.34 + 0.000038x | y = -0.5 + 0.027x |
| $R_m$ | y = -0.26 + 0.031x | y = -0.72 + 0.1x | y = -0.22 + 0.017x | y = 1.6 + 0.074x |

***Figure S5- 7:*** *Experimental design failures in the root exclusion collars. Roots were penetrating the exclusion collars via the open bottom of the collar of through the meshed windows.*

# Chapter 6: Conclusions

The purpose of this study was to measure the soil respiration and the bioavailability of soil nutrients in a mature temperate woodland during the first two years of $eCO_2$ enrichment to +150 ppm above ambient conditions. This is the first time that belowground responses of soil $eCO_2$ fluxes and nutrient dynamics of mature temperate woodland are under high-resolution scrutiny, both temporally and spatially. Also, this is the first time soil respiration was partitioned to its respiratory sources in a forest FACE experiment. We had the opportunity to study the combined effect of eCO2 and extreme events on belowground responses, providing a unique insight into complex interactions at an ecosystemic level. Moreover, this study provided the advantage to study the responses of the woodland under the 2050 atmospheric $CO_2$ scenario as a coetaneous event.

Soil respiration was higher under $eCO_2$ for both Year 1 and Year 2 (~21 and 36%, respectively), and the change was more prominent in Year 2. The temperature sensitivity $Q_{10}$ was decreased by 30 and 12% for Year 1 and 2, respectively, under $eCO_2$. Bioavailable nitrate was decreased by 27 and 15% under $eCO_2$ during years 1 and 2, respectively, while there was no difference in ammonium. Moreover, phosphate had a 10% decrease during Year 1 and a 5% increase during Year 2 under $eCO_2$. Furthermore, there was a ~50 and 8% increase in microbial biomass carbon in the O and B horizons, respectively, whereas there was a 15% decrease in microbial biomass carbon in A horizons under $eCO_2$. Under $eCO_2$ microbial biomass nitrogen increased by 15% in the O horizon but decreased by 2 and 17 %at A and B horizons. A similar pattern was observed for microbial biomass phosphorus, with the O horizons having a 53% increase under $eCO_2$, while there was an 8 and 16% decrease in A and B

horizons. Lastly, both soil inorganic ammonium and nitrate decreased under $eCO_2$, with the decrease being intensified with depth.

Moreover, soil respiration during Year 2 was decreased by 47 and 40% under ambient and $eCO_2$ conditions, respectively, compared to Year 1. Contrastingly, all soil bioavailable nutrients' concentrations were higher during Year 2 under both ambient and $eCO_2$ conditions. Ammonium availability increased by 47 and 49% under ambient and $eCO_2$ conditions, respectively. Similarly, nitrate availability increased by 70 and 101% under ambient and $eCO_2$ conditions, respectively, during Year 2. Sequentially, phosphate availability during Year 2 increased by 100 and 133% under ambient and $eCO_2$ conditions, respectively.

During the first year of the experiment, although the ecosystem was still in flux, $eCO_2$ stimulated the total soil respiration. Both the autotrophic and heterotrophic components were stimulated under $eCO_2$, with the heterotrophic component having higher fluxes. Moreover, root respiration was lower in $eCO_2$ arrays, while hyphal respiration was higher in $eCO_2$ arrays.

The soil respiratory components had different sensitivities towards the abiotic factors (soil temperature and soil moisture), and $eCO_2$ was observed to alter those sensitivities. Thus, shifts in the relationships between soil respiratory components and abiotic factors due to $eCO_2$ can significantly impact the C cycle and the ecosystems' potential of sequestering C in the long-term.

# Chapter 7: Future Outlook

This work highlights the importance of long-term, large-scale climatic experiments. Belowground responses can vary considerably; thus, rigorous and prolonged monitoring is required to make accurate predictions and take the correct steps for mitigation. Moreover, this work showcases that although field techniques are well established, in reality, they have multiple drawbacks and are not designed for long-term monitoring. Further work is required to focus on ensuring sufficient, cost-effective field-monitoring methods that can address a plethora of research questions at multiple temporal and spatial scales in the forest soil. Thus, it is pivotal to explore new techniques of partitioning soil respiration *in situ* in as non-destructive ways as possible. Failing to do so, a significant component of the global C cycle will remain highly uncertain.

Moreover, the $eCO_2$ interaction with abiotic factors and their effect on soil respiratory components remains insufficiently understood. Further work to determine individual responses is required, as well as simultaneous investigation of the respiratory components' combined responses is necessary to fully understand belowground C pools and sources and their potential shifts under $eCO_2$. Tackling the C budget uncertainties necessitates a deeper understanding of root, hyphal and microbial dynamics.

Furthermore, extensive work is required in the deeper soil horizons in order to be able to identify potential nutrient leaching losses through the ecosystem as well as potential shifts in microbial biomass. This will enable a more thorough understanding of soil biogeochemistry under $eCO_2$ to be ascertained and more effective model predictions and, thus, management strategies to be implemented.

Moreover, a more thorough investigation of the soil microbial and fungal community is required. There is still very little known about the communities' responses under $eCO_2$, and the scarce datasets show variable responses. The shifts in dominance of certain microbial and fungal species, the relative abundance and richness of microbial communities might have significant effects on the soil C cycle and the aboveground communities.

Another essential aspect that affects the soil cycle, and there is limited knowledge about the potential $eCO_2$ effect, is nutrient availability. Research is predominantly focused on nitrogen and phosphorus responses; however, availability and turnover rates of other macro-, micro-nutrients, and trace metals can significantly impact ecosystem functioning if biogeochemical cycles are observed to be decoupled under $eCO_2$.